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Africa

Intra-annual to multi-decadal xylem traits in a tropical moist semi-deciduous forest of Central Africa

ir. Tom De Mil

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences:

Forest and Nature Management

Dutch translation of the title: Seizoenale tot multidecadale xyleemeigenschappen in een vochtig semi-bladverliezend bos in Centraal-Afrika

(Front cover: view of the Nkulapark in the Luki reserve, with an X-ray CT image of a tree core, back cover: concept drawing of a cored tree stem, with one section representing live monitoring of wood via pinning, the other section representing xylem traits)

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Preface

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Samenvatting

Een groot deel van de landelijke oppervlakte op aarde is begroeid met tropische bossen. Toenemende menselijke druk doet hun aandeel afnemen en hun structuur wijzigen. Bovendien zijn huidige tropische bossen onderhevig aan klimaatsveranderingen. Ze spelen een sleutelrol in de koolstofbalans van de aarde, omwille van hun vermogen om koolstof te capteren in biomassa. Het is essentieel om de evolutie van tropische bomen, die in gewijzigde milieuomstandigheden zullen moeten leven, te voorspellen. De stam draagt voor het grootste deel bij tot de totale boombiomassa. Biomassa opslag gebeurt via het vormen van irreversibele groeilagen van hout (i.e. xyleem), dat aanleiding geeft tot radiale groei (i.e. diktegroei). Het monitoren van radiale groei gebeurt door permanente proefvlakken in het bos waarbij de diameter op borsthoogte van de bomen herhaaldelijk wordt gemeten, maar deze proefvlakken gaan niet verder terug dan enkele decennia waardoor gedetailleerde kennis van groei op lange termijn ontbreekt. Het hout zelf is een sleutelgetuige van de omgeving waarin de boom groeit. In bomen uit gematigde streken zorgt de afwisseling van lente en herfst voor groeiringen die duidelijk zichtbaar zijn op een doorsnede van een stam: jaarringen. Jaarringanalyse of dendrochronologie verkent dit natuurlijk archief door accuraat de breedte van jaarringen te meten en te synchroniseren. Dit laat toe om de leeftijd van bomen te bepalen en het klimaat uit het verleden te reconstrueren.

Bij veel tropische bomen zijn ook groeiringen zichtbaar in het hout. Deze ringen zijn vaak minder uitgesproken en ontstaan doorgaans door de afwisseling van droge en natte periodes. Jaarringanalyse van tropische bomen is in sommige gevallen succesvol om lange termijn groeipatronen te achterhalen. De techniek werd echter oorspronkelijk ontwikkeld voor gematigde streken, waardoor veel studies problemen vermelden met het dateren van hout via de klassieke aanpak van ringbreedtes, met grote onzekerheden tot gevolg. Er zitten immers veel meer kenmerken in hout dan enkel ringbreedte. Door binnen de ringgrenzen te kijken naar gedetailleerde xyleemeigenschappen zoals het patroon van vaten (die zorgen voor watertransport), parenchym (bescherming tegen schade en pathogenen) en vezels (mechanische stabiliteit), kunnen we de subtiele boomrespons in kaart brengen.

Om de mechanismen en de timing achter de vorming van hout te achterhalen, is het nodig het aspect van fenologie (i.e. de studie van wederkerende fenomenen) te belichten op het niveau van het primaire meristeem (knoppen) en het secundaire meristeem (vasculair cambium). Vooral in de tropen is dit aspect essentieel, omwille van een hoge variatie in fenologie, die de uiteindelijke houtvorming zal beïnvloeden.

Dit doctoraat graaft in het xyleemarchief door verder te kijken dan de klassieke jaarringanalyse. Wij onderzoeken intra-annuele (binnen één jaar) tot multidecadale (meerdere decennia) xyleemeigenschappen op verschillende tropische soorten in het Luki UNESCO Man and the Biosphere reservaat, dat deel uitmaakt van een semi-bladverliezend tropisch bos aan de rand

van het Mayombewoud, nabij de Atlantische Oceaan (westen van de Democratische Republiek Congo). Het studiegebied werd geselecteerd omwille van de grote fenologische variatie binnen en tussen soorten.

In dit doctoraat ligt de focus vooreerst op *Terminalia superba*, een boomsoort met duidelijke jaarringen, maar met aanzienlijke variatie in bladzetting en xyleemkenmerken tussen de jaarringgrenzen. Gedurende twee groeiseizoenen werden bladfenologie, stamfluctuaties en cambiale fenologie gemonitord. Verschillen in aanzet van bladvorming van 45 dagen werden opgetekend tussen bomen. Deze verschillen zijn persistent tussen jaren en onafhankelijk van regenval. Dit resulteert in boom-specifieke tijdstippen van houtvorming binnen de soort. De ringen die werden gevormd gedurende deze seizoenen werden onderworpen aan cellulose isotoopanalyse en houtanatomische metingen. Verschillen in cambiale fenologie resulteren in verschillende tijdskaders waarbinnen de bomen groeien. Omwille van deze verschillen zouden xyleemkenmerken tussen bomen moeten vergeleken worden op een intra-annuele tijdsas, eerder dan uitgemiddeld te worden binnen een inter-annueel tijdskader.

In een volgende sectie stellen wij X-stralen tomografie densitometrie voor als een techniek om intra-annuele xyleemkenmerken op een uniforme en snelle wijze te schatten, via gedetailleerde metingen van het volumegewicht. Het resultaat hiervan is een densiteitsprofiel waarop ringgrenzen, maar ook intra-annuele variaties te zien zijn. In tropische bedektzadige boomsoorten zoals *T. superba,* bemoeilijkt de aanwezigheid van vezels, parenchym en vaten de interpretatie van een densiteitsprofiel. Vandaar dat een ontbinding van het profiel in zijn anatomische componenten noodzakelijk is. Voor deze soort is het aangetoond dat vaten en parenchym het volumegewicht verlagen, terwijl vezelwandfracties de densiteit verhogen. Langsheen de jaarring eindigt de gestaag stijgende vezelfractie abrupt op de jaarringgrens. X-stralen tomografie densitometrie is niet alleen een geschikte methode om deze ringgrenzen te definiëren, maar bovendien kunnen de intra-annuele profielen direct gelinkt worden aan intra-annuele houtkenmerken in *T. superba*.

Nadien worden bovenstaande technieken gebruikt op een waaier aan soorten in de Mayombe, van zowel de onderetage als de bovenetage. Kennis over intra-annuele houtvorming in tropische bomen is beperkt, vooral van de onderetage. Het cambium van 4 onderetage soorten werd via cambial pinning gemonitord, waarna een Gompertz groeimodel aan de groeiwaarden werd gefit. Een soortspecifieke respons werd geobserveerd, maar het merkwaardige is dat veel van de bomen in de dataset geen xyleem vormden doorheen het hele groeiseizoen, behalve wond-geïnduceerde xyleemvorming op de plaats van de pinning. Intra-annuele variabiliteit in groei illustreert de verschillende responsen van soorten en individuele bomen op het milieu. Bloei- en bladfenologie zouden deze verschillen kunnen verklaren, maar standplaatsverschillen en invloed van omstaanders zouden ook beter moeten verklaard worden.

Vervolgens werd overgegaan van het intra-annuele naar het multidecadale niveau waarbij xyleemeigenschappen werden onderzocht met een unieke dataset van herontdekte bomen van het Nkulapark, een oud boomgroei - en fenologie observatie park van het Luki reservaat. Jaarlijkse diametermetingen van 4767 bomen, struiken en lianen van 176 soorten werden gemeten van 1948 tot 1957. Deze werden geanalyseerd en vergeleken met de 414 bomen die teruggevonden werden in 2014. Bovendien dragen deze bomen een nagel met nummerplaatje uit 1948, dat een uniek spoor nalaat in het hout, zodat het xyleem ondubbelzinnig kan gedateerd worden. 134 stalen van 18 soorten werden genomen naast het nagelspoor. De gemiddelde radiale groei is heel variabel tussen soorten, sommige soorten zijn 30 cm gegroeid, terwijl sommige onderetage bomen niet gegroeid zijn sinds 1948. Als naar het xyleem gekeken wordt, dan zijn ringstructuren duidelijk op nagenoeg alle bomen, maar het gemiddeld aantal ringen bedroeg slechts 32, terwijl er 66 jaar verstreken is. Dit was niet alleen het geval voor de onderetage, maar ook voor de bovenetage, waar er duidelijke radiale groei is op basis van de nagelsporen en de diametermetingen, maar dit valt niet af te leiden uit het aantal ringen. De lage groei van de onderetage, doet vermoeden dat de oudste bomen wellicht niet de woudreuzen zijn, maar eerder de kleinere onderetage bomen.

De laatste sectie van dit proefschrift omvat een discussie om zogenaamde anomalieën in klassieke jaarringanalyse (ontbrekende ringen, onduidelijke ringen), eerder te gaan zien als een opportuniteit voor de studie van xyleemeigenschappen op een breed spectrum van tropische bomen. Dit moet dan bekeken worden vanuit een equatoriaal standpunt, waarbij zogenaamde ring-anomalieën, eerder de regel zijn dan de uitzondering.

De belangrijkste resultaten van dit proefschrift zjin:

- Het bewijs dat binnen een tropische soort met een gekend jaarringpotentieel zoals *Terminalia superba*, er aanzienlijke fenologische verschillen zijn die leiden tot een verschuiving van xyleemeigenschappen op de tijdsas.
- Ontbinding van het intra-annueel densiteitsprofiel in zijn xyleem anatomische componenten toont aan dat X-stralen tomografie een geschikte techniek is om intra-annuele xyleenkenmerken te gaan beschrijven.
- De ontwikkeling van een X-stralen tomografie toolchain, die toelaat om veel stalen te scannen in een korte tijd, alsook om op een formele basis profielen te matchen op basis van intra-annuele densiteitsprofielen, en dus intra-annuele xyleemkenmerken.
- Het aantonen, via een lange termijn experiment dat verschillende boomsoorten in de Mayombe duidelijke ringen vertonen, maar dat het aantal van die ringen zelden overeenkomt met het aantal jaar dat verstreken is. Bovendien wordt aangetoond dat sommige bomen geen xyleemgroei vertonen over een periode van 66 jaar.

Traditionele tropische jaarringanalyse kan alleen toegepast worden op een beperkt aantal soorten, terwijl permanente proefvlakken wel de ecologische integratie van alle soorten

omvatten, maar niet met een hoge precisie. Daarom zouden de xyleemeigenschappen beschreven in dit werk, omwille van zowel hun intra-annuele en multidecadale tijdspanne die ze omvatten, verder uitgewerkt moeten worden om koolstofvoorraad van bomen te kwantificeren in tropische bossen.

Summary

Tropical forests cover a large patch of land on Earth's surface. Forests are disappearing or changing in structure due to increased human pressure. Furthermore, current forests are prone to climatic changes, and as a major sink for carbon they have a vital role in Earth's carbon balance. Given the fact that these forests are pushed into an unprecedented environment, quantifying and predicting the evolution of tropical forests to future climate change is urgent. The trunk contributes to the largest part of tree biomass. Biomass gain in the trunk occurs through the formation of irreversible growth layers of xylem (wood), which causes radial growth. Live monitoring of radial growth occurs through permanent sample plots, though they do not reach further than a few decades and detailed knowledge on xylem growth and carbon gain cannot be assessed. A key witness of the tree's past conditions is the xylem archive. In extratropical trees, the alteration of winter and summer induces alterations of xylem growth followed by dormancy, which forms clear visual rings in the xylem. Tree-ring analysis explores the xylem archive through detailed counting, measuring and synchronizing (i.e. cross-dating) ring widths in the xylem and allows to estimate tree age, assess historic tree response, and reconstruct past climate conditions. In tropical trees, many trees form rings in the xylem but these are more subtle and mainly caused by alternation of dry and rainy conditions. Tree-ring analysis has been shown successful for matching ring-width sequences in tropical trees, revealing long-term growth patterns and age of tropical trees. However, originally designed for extratropical trees, many ring-width based dendrochronological studies in tropical trees report difficulties in cross-dating, which results in discarding many samples, and causing large uncertainties, with many species being ignored. There is, however, more than only ring width to take into account. Looking between the ring boundaries, intra-annual xylem traits are of chemical and structural nature and provide us with seasonal information of tree response. In order to have a mechanistic understanding of when these traits are formed, knowledge of recurring events of meristems is essential: leaf phenology (buds) and cambial phenology (vascular cambium). Xylem traits from pith to bark are increasingly being explored to map tree performance in the past. In tropical trees, linking xylem structure to the past is less evident and depends on a thorough assessment of leaf and cambial phenology.

Thus, this dissertation aims at exploring the xylem archive beyond the assessment of tree ring boundaries in traditional tree ring analysis. We investigate intra-annual to multi-decadal xylem traits on tree species from the Luki UNESCO MAB Biosphere Reserve, which is part of a semi-deciduous tropical rainforest at the southernmost edge of the Mayombe forest near the Atlantic Ocean (western DR Congo). This study site is of special interest due to the presence of inter- and intra-species variation in leaf and cambial phenology.

Firstly, we focus on *Terminalia superba*, a tree species with a known dendrochronological potential, but with large variations in leaf phenology and a considerable variation in intra-annual xylem traits such as density fluctuations between the tree ring boundaries. During two growing

seasons, leaf phenology, diel stem diameter variations, and cambial phenology were monitored to disentangle tree response. Leaf onset differences up to 45 days were observed between trees and persisted between years, irrespective of rainfall onset, resulting in tree-specific xylem onsets. The rings that were formed during monitoring were subjected to intra-annual wood cellulose isotope and wood anatomical measurements. Differences in cambial phenology result in tree-specific timeframes of growth, and the resulting intra-annual traits should be compared between trees on an intra-annual time axis, rather than a distance axis within an inter-annual timeframe.

Secondly, X-ray Computed Tomography (X-ray CT) microdensitometry is proposed as a technique to assess intra-annual traits in a uniform and fast way. In tropical angiosperm tree species such as *T. superba*, the presence of fibres, parenchyma and vessels complicates the interpretation of the density profile. Therefore, a decomposition of intra-annual profiles into its anatomical components was necessary and showed that vessels and parenchyma decrease wood density, whereas fibre wall fractions, and proportion positively affect wood density. The slowly increasing fibre wall fraction along the development of the tree ring abruptly ends and delineates the tree ring boundary. X-ray CT microdensitometry is not only an appropriate method to define tree ring boundaries, but the resulting intra-annual density profiles were proven to be directly linked to intra-annual wood traits in *T. superba*.

Thirdly, to upscale the technique, we present an X-ray CT microdensitometry toolchain of tree increment cores, resulting in profile datasets suitable for visual exploration as well as density-based pattern matching. A digital workflow allows to generate density profiles of large sets of cores in a short time span that provide sufficient intra-annual density information for tree-ring analysis. Density-based pattern matching is able to formally detect anomalies in ring series that can be corrected via interactive software. Furthermore, visual exploration of such datasets is of high value, as patterns emerge of trees that have similar intra-annual information in their rings. The dated profiles can be used for high-resolution chronologies and also offer opportunities for fast screening of lesser studied tropical tree species.

Subsequently, we expanded the above methods to multiple species of the Mayombe, both understory and canopy species. Knowledge on intra-annual xylem growth remains understudied in tropical regions, especially for understory species. The cambium of 4 species was monthly marked at the stem base via the pinning method. In many cases, intra-annual growth was successfully derived and could be fitted with a Gompertz function. A species-specific response is observed, which is assumed to be due to phenology differences, but variation between trees of the same species is observed as well. Anomalies such as circumferential variability and wedging were reported, but the most remarkable result is that many of the trees in the dataset had no xylem formation at the stem base, throughout the entire season. Intra-annual variability in growth illustrates the different responses of species and individual trees to environmental drivers. Leaf phenology might explain the differences, although site and influence of other trees

should be considered as well. A large number of trees show no xylem growth at all, apart from wound-induced local growth.

Going from the intra-annual level to the multidecadal level, xylem traits were assessed by the use of a unique set of rediscovered trees from the Nkulapark, an old forest growth and phenology monitoring park. Records of yearly diameter data of 4767 trees covering 176 species measured from 1948 to 1957, were digitized and analysed, and compared to 414 trees that were rediscovered in 2014. The diameter of the rediscovered trees was measured and the nails carrying the original 1948 number tag serve as a cambial timestamp in the wood, an unprecedented opportunity to directly assess wood formation. 134 samples from 18 species were taken, containing the nail marks in order to analyse radial xylem growth. The average radial increment over the 66-year timespan was highly variable: some understory trees showed no radial increment at all, while other canopy species showed up to 30 cm radial growth. When the xylem structure was examined, ring structures were visible on all species but it was shown that in some cases only few discernible rings were formed in understory trees. Some canopy trees showed considerable radial growth, had rings that concur with the calendar years elapsed and thus give a reliable view on the tree's past. Additionally, canopy tree species were found with radial growth, but the number of rings did not concur with the years elapsed. The low increment of understory species, can also be a reason that the oldest tropical tree should not only be sought among the tallest trees. Anomalies were thus detected on the intra-annual scale, and confirmed at the multi-decadal scale.

In the last section of this dissertation, a discussion is held to reconsider these anomalies as an opportunity for trait measurements on a wide range of tropical trees. Instead of discarding samples with irregular or missing rings, they should be further investigated via xylem traits from an equatorial viewpoint, rather than applying tree-ring analysis on a limited set of species.

The main outcomes of this dissertation are:

- Evidencing that within a tropical species with a known dendrochronological potential such as *T. superba*, phenology differences lead to considerable shifts in xylem traits along a time axis.
- Decomposing the intra-annual density profile into its xylem anatomical components shows that X-ray CT densitometry allows to assess intra-annual traits.
- Developing an X-ray CT densitometry toolchain while developing a formal method to synchronize density profiles based on density information.
- Demonstrating that several species in the Mayombe show distinct tree ring boundaries, but the number of discernible rings seldom matches the number of elapsed years. Moreover, some trees appeared to have no xylem growth at the stem base after 66 years.

Traditional tropical tree-ring analysis can only be performed on a limited set of species, while permanent sample plots do provide the ecological integration of all species, but can only be assessed in periodical terms. Xylem traits, whether or not measured via X-ray CT densitometry, are scaled from the intra-annual to the multi-decadal level and should therefore be further explored to evaluate carbon stocks through time in tropical forests.

List of abbreviations

ANCA	Automated Nitrogen Carbon Analyzer
DBH	Diameter at Breast Height
DBPM	Density-Based Pattern Matching
DR Congo	Democratic Republic of the Congo
GUI	Graphical User Interface
IADF	Intra-Annual Density Fluctuation
INEAC	Institut National pour l'Etude Agronomique du Congo Belge
INERA	Institut National pour l'Etude et la Recherche Agronomique
IRMS	Isotope Ratio Mass Spectrometer
KNN	K-Nearest Neighbour Algorithm
NSC	Non-Structural Carbohydrates
MAB	Man and the Biosphere, progamme by the UNESCO
MXD	Maximum Latewood Density
QWA	Quantitative Wood Anatomy
RG	Relative Growth
RMCA	Royal Museum of Central Africa
SDV	Stem Diameter Variations
TWS	Tree Water Status
UNESCO	United Nations Educational, Scientific and Cultural Organization
WD	Wood Density, specific gravity
X-ray CT	X-ray Computed Tomography

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General introduction

1.1 Tree age, response and tree rings in tropical forests

Forest trees contribute to about 90% of the terrestrial biomass of carbon (Klein & Hoch, 2015) and tropical forests hold about half of this biomass (Pan *et al.*, 2011; Hunter *et al.*, 2013). As a major sink for carbon under threat (Holm *et al.*, 2017), quantifying and predicting the evolution of tropical forests to future climate change is essential and can be done by large scale networks of permanent sampling plots, where stem radial growth, mortality and recruitment are monitored (Lewis *et al.*, 2009, 2013; Brienen *et al.*, 2015). Detailed knowledge on long-term radial growth rates in tropical trees remains scarce nonetheless (Clark *et al.*, 2007; Worbes *et al.*, 2003). Moreover, it is important to know tree growth and age of tropical trees (Chambers *et al.*, 1998) in function of tree ecology, and aforementioned future tree response and carbon storage. The major part of carbon gain occurs through radial stem growth via wood formation (Sass-Klaassen, 2015). Therefore, understanding the mechanisms of wood formation is essential for carbon cycling (Cuny *et al.*, 2015) and carbon accumulation over time. Nevertheless, a detailed assessment of wood formation in tropical trees is clearly understudied compared to extratropical trees. Importantly, wood formation can be observed via growth rings, an archive that remains underexploited.

In its quest for resources to survive, a tree develops from a seedling to an adult by combining height growth and radial growth, meanwhile maintaining mechanical stability, providing upward water transport, metabolism and downward sugar transport. Radial growth is caused by cell layers produced around the circumference of the stem by a cell division zone called the vascular cambium (Phillipson *et al.*, 1971; Larson, 1994). Towards the outside, the vascular cambium produces phloem cells to allow for downward sugar transport and to form bark. Towards the inside, xylem cells are formed, the basic component of wood, providing upward water transport and stability. In temperate and

boreal regions, an interruption in the xylem radial growth occurs every year: by the end of the growing season, photosynthetic activity decreases, and temperature and day length cause many trees to eventually shed their leaves (Singh *et al.*, 2017), resulting in a complete dormancy of the cambium, and eventually, cessation of wood formation. Increase of temperature and day length in the following spring allows for bud break (Körner & Basler, 2010) to restart photosynthesis and start new wood formation when sufficient water is available: cells are formed and the process repeats. The difference in growth rate of cells between the beginning and the end of the growing season can be seen as rings on a cross section of the stem. The tree ring width is a proxy of the amount of wood formed in that year, and can vary from year to year, which forms the basis of dendrochronology and determination of tree age (Fritts, 1976; Speer, 2011). A tree ring is a combined result of intrinsic and environmental factors, which makes them a natural archive of the past (Schweingruber, 1996; Vaganov *et al.*, 2006). Tree rings play an important role in annually resolved climate reconstructions (Briffa *et al.*, 1998; Esper *et al.*, 2012), as well as in assessing carbon dynamics, ranging from seasonal to decadal scales (Babst *et al.*, 2014). Because of the pronounced seasonality causing clear ring structures, tree-ring analysis is historically developed for trees of temperate, boreal and arctic climate regions.

In tropical latitudes, photoperiod and temperature can be more or less constant (Fromm, 2013) and annually recurring drought (and to a lesser extent, periodic flooding, Worbes & Junk, 1999) is the main driver for periodical wood formation (Worbes, 2002; Brienen & Zuidema, 2005). Tropical tree-ring analysis is a relatively young discipline, with some early exceptions (Brandis, 1898; Geiger, 1915; Coster, 1927; Berlage, 1931). Recent years, studies investigating tree ring potential, and thus, periodical wood formation, exist for a plethora of species (review: Worbes, 2002), and actual determination of climate response and age of trees is possible using classic tree-ring analysis (Brienen & Zuidema., 2005; Schöngart *et al.*, 2006; Groenendijk *et al.*, 2014).

Thus, in the past few decades, several discussions on tropical tree-ring analysis have led to showing the unambiguous presence of annual rings throughout many regions in the world.

However, many of these studies mention low correlations with climate, and the difficulty of cross-dating (i.e. the matching of ring sequences between trees) causes that tropical tree ring analyses still face some serious challenges, compared to temperate regions. Some studies in the tropics generate poor individual synchronized ring width patterns (up to 50%, Schöngart *et al.*, 2006) and many samples are rejected (Brienen & Zuidema, 2005).

Nevertheless, due to future increased droughts (Holm *et al.*, 2017), knowledge of detailed radial growth via tree-ring analysis is essential and urgent in predicting how these forests will respond and able to maintain their function as a carbon sink.

There is, however more than only tree ring width to derive environmental information from (Fonti *et al.*, 2010; Battipaglia *et al.*, 2014; Sass-Klaassen, 2015). Tree ring width is merely an integration of information over one year (Fritts, 1976), neglecting the seasonal information that is present between these ring boundaries (Fonti *et al.*, 2010).

1.2 Intra-annual wood traits

To assess seasonal information in tree rings, the aspect of traits is key. Several definitions of traits are discussed and can be considered to be measurable, morphological, biochemical, physiological and phenological features on individuals or components of the individual, that can be quantifiable on a determined time axis (Beeckman, 2016). As a simplification, wood traits are classified as chemical and anatomical traits (Eckstein, 2004).

1.2.1 Chemical traits

Xylem cells consist predominantly of carbon (C), oxygen (O) and hydrogen (H) that are photosynthesized to chemical compounds such as cellulose, hemicellulose and lignin. Cellulose and hemicellulose are made in the vascular cambium from sugars and starch via the intermediate non-structural carbohydrates (NSC). NSC are immediately deployed for wood formation, as such irreversibly incorporated in the wood structure (Würth *et al.*, 2005) or can be stored in a reserve pool of the tree to provide leaf, root and stem biomass. Lignin is synthesized in the xylem cells themselves during the cell differentiation process. Other components, such as extractives are non-structural, more complex compounds such as resins, gums present in the wood, that serve various roles in terms of protection against pathogens (Fengel & Wegener, 1984).

During synthesis of chemical compounds, various ecophysiological and meteorological mechanisms will alter the isotope ratio of C ($\delta^{13}C$), O ($\delta^{18}O$) and H (δD) (Managave & Ramesh, 2012). The isotope ratio of source water ($\delta^{18}O$, δD) and CO_2 ($\delta^{13}C$) undergoes, depending on drought, relative humidity, irradiation and temperature, several mixing and fractionation processes before they are incorporated in wood, and offer us a deeper insight

in plant physiology. For a more extensive description of these fractionation processes from leaves to wood, the reader is referred to Managave & Ramesh (2012), Helle & Schleser (2004) and McCarrol & loader (2004). The isotope composition can be measured in the biomass of leaves (Helle & Schleser, 2004), in NSC (Scartazza *et al.*, 2013) and in the xylem, and the latter forms the basis of stable isotopes analysis of tree rings (McCarrol & Loader, 2004).

Analysis of stable isotopes in wood is commonly done on extracted wood, thus eliminating extractives, hemicellulose and lignin that can blur the signal, which leaves cellulose as only component to be analysed, because it is a component formed during cell expansion in the secondary wall (McCaroll & Loader, 2004; Gessler *et al.*, 2014). However, bulk wood stable isotope analysis showed little differences compared to cellulose stable isotopes in some studies (Harlow *et al.*, 2006).

Cellulose stable isotopes $\delta^{13}C$ and $\delta^{18}O$ are of special interest in the tropics, as a proxy for physiological processes such as stomatal conductance, intrinsic water use efficiency (Van der Sleen *et al.*, 2014) and source water use. $\delta^{13}C$ is suitable in regions where moisture is limiting, as the isotope discrimination depends on stomatal aperture (Gebrekirstos *et al.*, 2009; Brienen *et al.*, 2016). $\delta^{18}O$ in the wood is related to the $\delta^{18}O$ composition of the source water and depending on the region (e.g. continental vs. coastal regions, see Dansgaard (1964)), is linked to the amount of precipitation (Schollaen *et al.*, 2014).

Intra-annual sampling investigates seasonal wood response in a detailed way (Evans & Schrag, 2004; Fichtler *et al.*, 2010; Schollaen *et al.*, 2014). Stable isotope analysis of wood has proven the annual nature of growth rings (Krepkowski *et al.*, 2013), and has resolved seasonality in tropical trees that lack visible ring boundaries (Poussart *et al.*, 2004; Anchukaitis *et al.*, 2008).

Although analyzing intra-annual cellulose stable isotopes is a tedious process, improvements on automatisation and sampling resolution are made (Schollaen *et al.*, 2014).

Next to the above mentioned organic compounds, anorganic compounds in the wood such as calcium can delineate tree ring boundaries in trees without discernible rings (Poussart *et al.*, 2006) and are also being explored as a high-resolution proxy for tropical wood species (Hietz *et al.*, 2015).

1.2.2 Wood anatomical traits

Xylem cells in trees are generally differentiated into three main types (Schweingruber, 1996; Schuetz *et al.*, 2013; Hacke, 2015; Rathgeber *et al.*, 2016); (i) conduits as vessels (angiosperms) and tracheids (conifers), (ii) axial/ ray parenchyma and (iii) fibres.

Conduits have a hydraulic function to transport water from the roots to the crown, dimensioned as a trade-off between hydraulic capacity and protection against embolism. Fibres in angiosperms have a mechanical support function. Parenchyma is linked to NSC content since storing starch (Hacke, 2015), and also serves as protection and interface between the cambium, heartwood formation and protection against embolism for vessels.

Although each of these types have multiple functions (Beeckman, 2016), the variation of the wood anatomy in a tree ring is a balance of these three competing functions under environmental and phylogenetic constraints (Chave *et al.*, 2009 in Fonti *et al.*, 2010). Wood anatomical variation across taxa reveals ecological information of species, as well as their adaptation to the environment they live in (Wheeler & Baas, 1993). Radial variation in vessel traits is due to tapering (Anfodillo *et al.*, 2006), but also year-to-year, and within-year variation is present due to environmental conditions. In the vascular cambium, cells follow a process of cell type determination, cell division, cell elongation and cell differentiation. Vessels and fibres die a programmed death after their formation, while parenchyma stays alive for multiple (Rathgeber *et al.*, 2016) up to 200 years (Spicer & Holbrook, 2007). These processes are genetically hardcoded, steered by phytohormones (Sorce *et al.*, 2013), but are influenced by environmental conditions as these cells are formed within a time window of days to weeks, thus directly imprinting this information in the wood. Consequently, wood anatomy is of special interest for tree response to climate, as anatomical variables will dynamically vary under changing environmental conditions (Carlquist, 2001; Wimmer, 2002; Fichtler & Worbes, 2012). Furthermore, severe drought periods could affect the rings for several years, as the carbohydrates (photo-assimilates) are depleted (Fonti *et al.*, 2010).

Especially in the tropics, delineation of tree ring boundaries is accomplished through careful description of wood anatomical variables: the 4 ring types from Worbes (1985), simplified from the classification of Coster (1927) are based on variations in size and frequency of parenchyma, fibres and vessels. The way in which these variables vary within the tree ring boundaries, offers opportunities to exploit this archive via quantitative wood anatomy (QWA) (Battipaglia *et al.*, 2010; von Arx *et al.*, 2016), revealing ecophysiological, climatological and ecological information of the past.

Wood anatomical variables are hard to measure in a fast way (Fichtler & Worbes, 2012), but developed software such as WinCELL (Regent instruments, Canada), ROXAS (Wegner *et al.*, 2013; von Arx & Carrer, 2014) and more general plugins in ImageJ (Schneider *et al.*, 2012) are already allowing to construct high-resolution chronologies.

However, the abovementioned techniques often are strongly dependent on surface preparations such as microtoming (Gärtner & Nievergelt, 2010) as well as sanding, and involve at least some degree of sample destruction. Therefore, additional proxies are searched for, such as detailed mapping of wood density via densitometry.

1.2.3 From wood anatomy to wood density: densitometry

Wood density (WD) is determined by chemical and wood anatomical traits (Lachenbruch & McCulloh, 2014). As a so called integrator variable (Chave *et al.*, 2009), WD is defined as ovendry mass divided by its volume (often expressed as kg m^{-3}) and can be seen as a proxy for carbon allocation per unit of volume (Müller-Landau, 2004; Plourde *et al.*, 2015). WD is a primary weighing variable in biomass estimations and both mass and volume vary with moisture content. Dry mass is used in any WD calculation, but the moisture content of the volume needs to be specified (Williamson & Wiemann, 2010).

WD values from literature are available for many species, and databases such as the global wood density database (Chave *et al.*, 2009; Zanne *et al.*, 2009) make these data easily accessible.

WD is an important indicator of the life history strategy of the tree (Müller-Landau, 2004; Swenson & Enquist, 2007; Zanne *et al.*, 2010) where there is a general negative relation between wood density and growth rate. WD also varies on a regional scale: In Neotropical forests, an average wood density value of 645 kg/m^3 was found, with decreasing values with increasing altitude and considerable variation at low-altitude sites (Chave et al., 2006).

Next to average WD per species, intra-tree WD trends from pith to bark are assessed for carbon (Henry *et al.*, 2010), ecology (Parolin, 2002; Woodcock & Sheer, 2002; King *et al.*, 2005; Nock *et al.*, 2009; Williamson *et al.*, 2012; Osazuwa-Peters *et al.*, 2014; Plourde *et al.*, 2015) and forestry studies (Montes *et al.*, 2007; Moya & Filho, 2009).

Classic wood density measurements (geometric method or water immersion) do not allow to measure small sections (Cook & Kairūkštis, 1990), or to arrive at intra- tree-ring level

resolution. There is an increasing interest in developing methods for determining wood density in a more detailed and less time-consuming way (Mannes *et al.*, 2007).

X-ray densitometry by means of radiographies was developed by Polge (1966) and is still widely used as a basic technique for densitometric profiling for dendroclimatic purposes. One of the most important variables derived is the Maximum Latewood Density (MXD) in boreal conifers, a key variable for climate reconstruction (Briffa *et al.*, 1998). The first attempt of X-ray based imaging for tropical angiosperm tree species was on *Aucoumea klaineana* by Mariaux (1967), to delineate tree ring boundaries in tropical wood, but showed difficulties due to vessel pattern and parenchyma banding. Annual growth rhythms have been detected with densitometry in the tropics (von Schnakenburg *et al*., 2008) and a climate-growth relation was shown by Akachuku (1985) on *Gmelinea arborea*. Despite the high resolution, the technique is costly and time-consuming. Recent efforts in specifically reducing labour and time costs are made by using high-frequency densitometry (Schinker, 2003; Wassenberg *et al.*, 2014). Nevertheless, the necessary surface treatment is still limiting and it is an indirect measurement as well.

X-ray Computed Tomography (X-ray CT) is increasingly used as a routine method in microscopy to observe the internal structure of objects in 3D (Dierick *et al.*, 2014). Increasing computing power allows to run the reconstruction algorithms necessary to convert 2D radiographies into 3D images. A generic set-up of an X-ray CT scanner is given in Fig. 1.1, with the X-ray source directed towards the object under study, which is rotating 360° on a rotation stage. The X-rays are attenuated by the object and are registered on the detector.

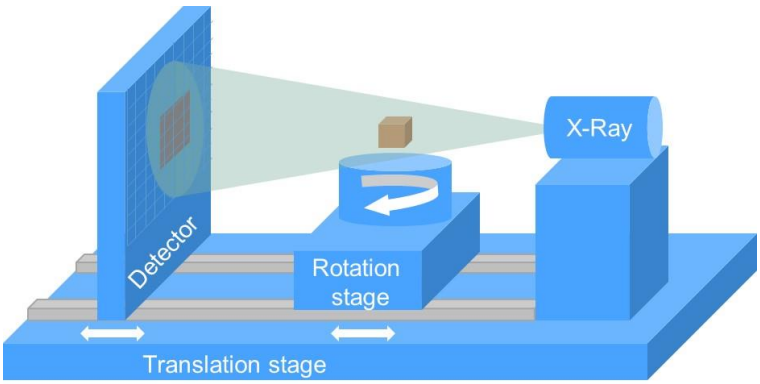

Figure 1.1 Generic set-up of an X-ray CT scanner.

Various types of scanners are developed and the resolution can range from below micrometer level for small samples (Trtik *et al.*, 2007; Van den Bulcke *et al.*, 2009) to millimetres for larger samples.

Vessel features can be delineated (Van den Bulcke *et al.*, 2014) and even the 3D structure of vessel patterns can be analysed (Brodersen *et al.*, 2011), making it a versatile tool to measure intra-annual wood traits as well as to perform tree-ring analysis (Steffenrem *et al.*, 2014). Furthermore, rescaling with a reference material that is scanned simultaneously, direct estimates of wood density are obtained (De Ridder *et al.*, 2011), making X-ray CT densitometry a promising tool for studying wood density variations in a detailed way.

1.2.4 Fixing xylem traits on a time axis: leaf and cambial phenology

The aforementioned improvements and measuring techniques allow to assess intra-annual wood traits at a higher level of detail than the tree ring. However, a mechanistic understanding of the influence of an environmental variable on a certain intra-annual trait in function of time remains elusive without knowledge of detailed timing of wood formation (Gričar & Rathgeber, 2011). A higher level of detail can simply not be assessed without knowledge on a narrower, seasonal timeframe. Therefore, intra-annual wood formation is needed to link intra-annual wood traits to their actual time of formation within the tree ring (Rossi & Deslauriers, 2007) in order to observe short-term adaptations to external factors (Steppe *et al.*, 2015).

In the following sections, phenology is defined as cambial phenology (onset and offset of intra-annual wood formation, Ziaco & Biondi (2016)) or as leaf phenology (leaf flushing and leaf shedding). The former occurs on the secondary meristem level (i.e. the vascular cambium), whereas the latter occurs on the primary meristem (i.e. leaf buds, flower buds) (Rossi *et al.*, 2013).

Recently it has been shown that cambial phenology and kinetics of tracheid development explain xylem structure of conifers (Cuny *et al.*, 2015; Rathgeber *et al.*, 2016). Unsurprisingly the knowledge on tropical angiosperm trees is known to a lesser extent, but experiments have derived the length of growing seasons as well as their climate sensitivity (Mariaux, 1967; Amobi, 1973; Fuji *et al.*, 1999; Krepkowski *et al*., 2011; Dié *et al.*, 2012; Trouet *et al.*, 2012; Morel *et al.*, 2015).

Several methods exist for monitoring cambial phenology (Fig. 1.2): (i) periodically wounding the cambium with a needle (Wolter, 1968), or by carving a rectangle in the bark (Mariaux, 1969)) causing a local growth cessation in the xylem (cambial pinning), followed by a harvest in order to measure the distance between the wounding and the cambial zone at time of felling. Relative growth is measured to take into account circumferential variation (Seo *et al.*, 2007). (ii) Periodically sampling the outermost wood section containing the last xylem ring, the cambium and the phloem (microcoring) to make wet microsections (Rossi *et al.*, 2006) is another method. Lignification and cell wall thickening can only be tracked using the latter method, yet it is less straightforward to determine relative growth (but see Rossi *et al.*, 2003; Van der Werf *et al.*, 2007; Harley *et al.*, 2012), which is conveniently done with cambial marking. Additionally, (iii) automatic radius dendrometers (De Swaef *et al.*, 2015) are used to monitor stem diameter variations (SDV) that can be logged from minute to monthly scale, providing information on reversible swelling and shrinking, as well as irreversible xylem growth. Information on water status can be derived as well (Zweifel *et al.*, 2001).

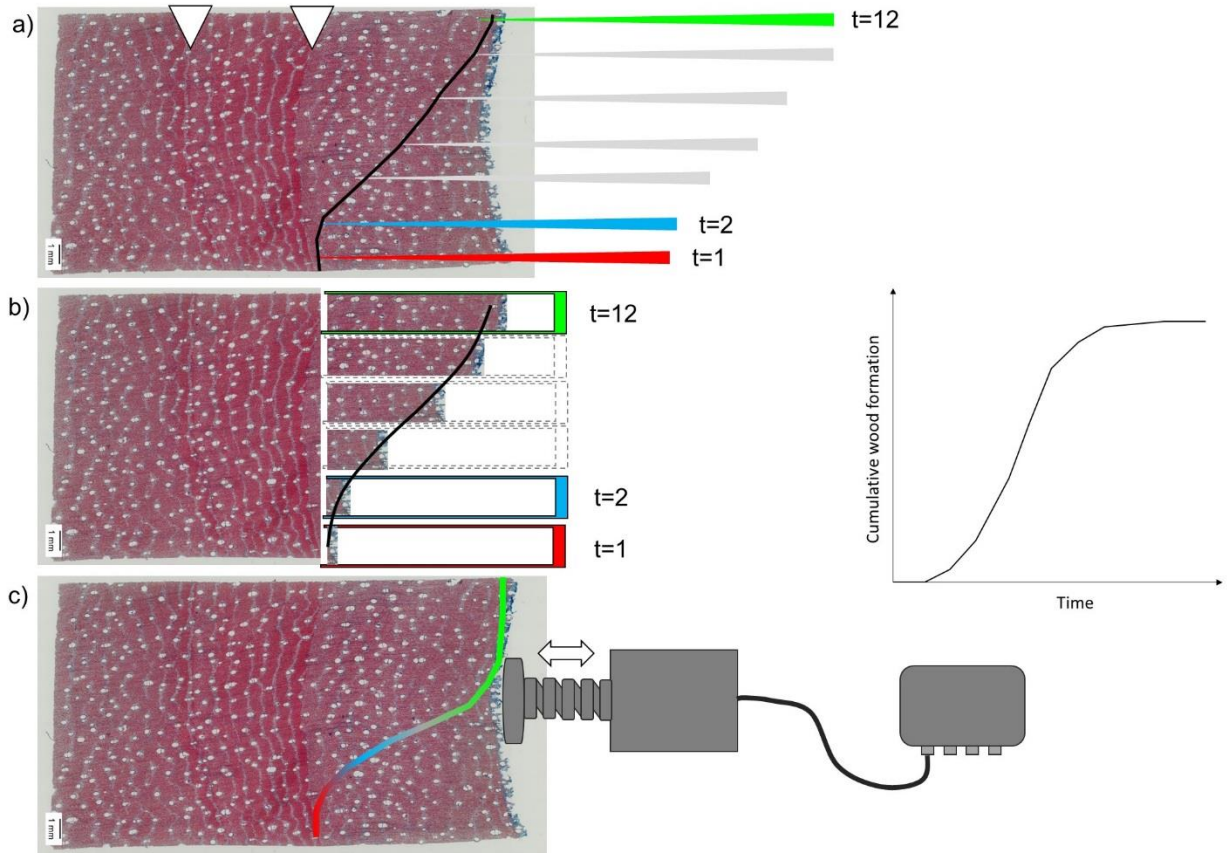

Figure 1.2 Illustration of the three main methods to derive cambial dynamics and seasonal growth during one season: (a) monthly cambial marking, (b) monthly microcoring and (c) automatic radius dendrometers. White arrows are the tree ring boundaries of the previous years

Monitoring SDV is essential for these cambial dynamics, revealing (i) physiological responses through stem water relations (Zweifel *et al.*, 2001; Steppe *et al.*, 2006) as well as (ii) irreversible, long term seasonal growth (Deslauriers *et al.*, 2003; Steppe *et al.*, 2006; Volland-Voight *et al*., 2011; Spannl *et al*., 2016), making it a key tool for assessing the tree response to environmental variables (Van der Maaten *et al.*, 2016).

The disadvantage is that shrinking and swelling cannot be disentangled from actual xylem increment on the seasonal scale. Therefore, a combination of the three abovementioned techniques should always be used (Mäkinen *et al.*, 2008). Other techniques that only measure cambial activity (i.e. difference between dormant season and growing season) can also be used using electrical resistance measurements (Smith *et al.*, 1984). Band dendrometers, repeated diameter measurements (Couralet *et al.*, 2010) and automatic circumference dendrometers are accurate, but when stem irregularities occur and a direct link with wood formation is sought, these methods are less suitable.

In the tropics, periodicity and intensity of droughts depend from region to region and high tree species diversity causes a high variability in tolerances and response strategy to these droughts (Worbes *et al.*, 2013). A main variable to assess this response strategy is leaf phenology (Borchert, 1999), and several tree types can be distinguished, ranging from evergreen to brevi-deciduous to deciduous (Borchert, 1994), occurring in the same forest (Borchert *et al.*, 2005) and related to functional groups (Couralet *et al.*, 2013; Wolfe & Kursar, 2015). In temperate and boreal regions, the effect of temperature and photoperiod on leaf phenology are clear, but in tropical and subtropical regions this relation is less evident (Singh *et al.*, 2017). Moreover, within-species variability at the individual level in phenology is important as well (Visser *et al.*, 2010; Forrest & Miller-Rushing, 2010).

Leaf and flowering phenology are key to describe tree development in the tropics (Borchert *et al.*, 2005), and, specifically leaf phenology is linked to tree water status (Borchert, 1999), able to describe cambial phenology (Iqbal 1994; González-González *et al.*, 2013; Mendivelso *et al.*, 2016; Morel *et al.*, 2015) and eventually tree-ring analysis (Enquist & Leffler, 2001).

Phenology is traditionally monitored *in situ*, with visual observations at the tree level (Panchen *et al.*, 2014) and also herbarium collections (Rivera & Borchert, 2001). Methodological efforts are made with near-remote sensing with timelapse cameras (Bater *et al.*, 2011; Sonnentag *et al.*, 2012). On a large scale and eventually global scale, phenology can be monitored using aerial observations (Borchert *et al.*, 2015) and satellite imagery (Ryan *et al.*, 2016).

1.2.5 Original contribution and thesis outline

Recent opinions and frameworks to disentangle tree response for the extratropics (Battipaglia *et al.*, 2014; Sass-Klaassen *et al.*, 2016) and the tropics in particular (Zuidema *et al.*, 2013) call for an integrative tree-based approach that combines several disciplines that deal with data ranging from minutes (ecophysiology), to months (phenology) to decades (dendrochronology and repeated diameter measurements). Especially in the Central African forests, knowledge on detailed wood formation and its coupling to eventual dendrochronology currently remains largely elusive (Gebrekirstos *et al.*, 2014). In general, there is a need for methodological improvements to assess intra-annual wood traits in a fast and reproducible way.

The main research question of this dissertation is how to assess and reveal the driving forces of the patterns of wood traits on pith-to-bark cross-sections in tropical trees. Additionally, can we date these wood traits, i.e. when are they exactly formed?

The Luki Man And Biosphere reserve in the D.R. Congo was chosen as research area due to its (i) position at the savannah- semi-deciduous forest boundary, with species that have dendrochronological potential, and other species that do not have such proven potential, depending on their functional behaviour; (ii) the availability of unique long-term growth data, allowing to assess tree growth over multidecadal scale, without the need of dendrochronology and (iii) the availability of long-term tree phenology data.

The Luki Reserve (33,000ha) is part of semi-deciduous tropical rainforest situated at the southernmost edge of the Mayombe forest near the Atlantic Ocean (western D.R. Congo) (Fig. 1.3).

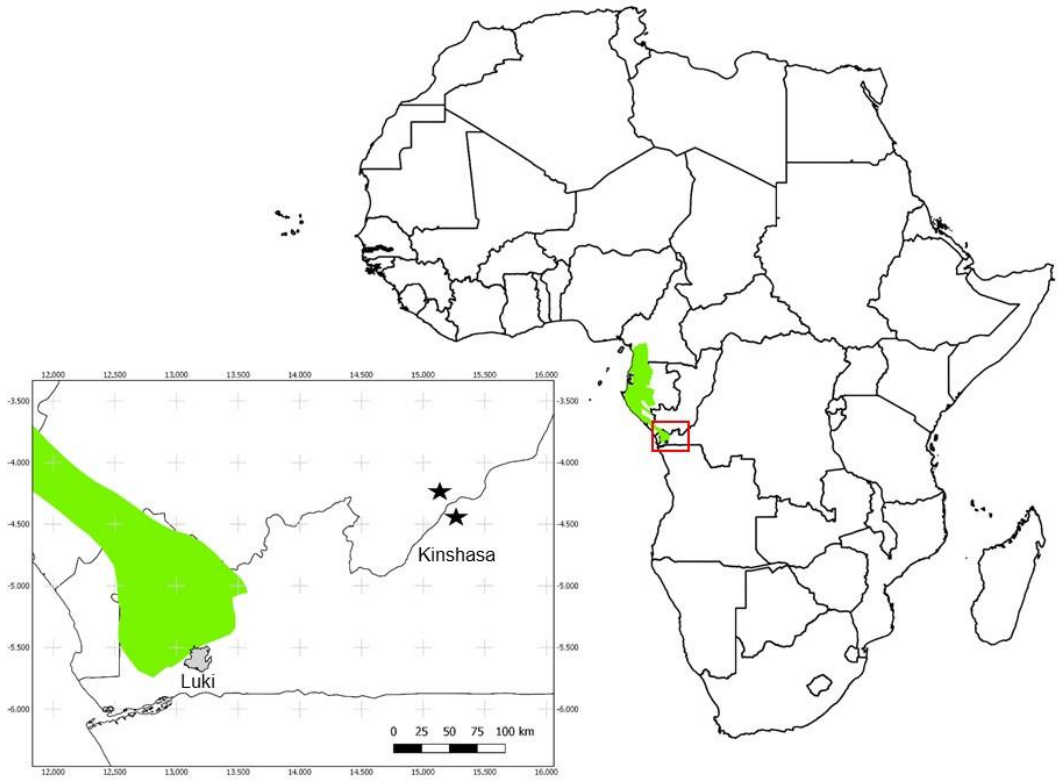

Figure 1.3 Location of the Luki Biosphere Reserve in the DR Congo, at the edge of the Mayombe. The depicted Atlantic Equatorial Coastal forest ecoregion delimitation is adopted from Olson et al. (2001).

As a Man and Biosphere reserve it is protected by the UNESCO since 1976. The Luki research station (5° 37.31' S, 13° 5.90' E) was founded in 1937 by INEAC (Institut National pour l'Etude Agronimique du Congo Belge) (Donis, 1948), and is since 1960 reformed to INERA (Institut National pour l'Etude et la Recherche Agronomique). The soils are generally determined as ferrallitic, acid and with poor chemical content (Sénéchal *et al.*, 1989).

June to September defines a distinct dry period, with a monthly precipitation of less than 50 mm (Fig. 1.4.). There is a small interpluvial during the rainy season, which can fluctuate from year to year (Sénéchal *et al.*, 1989) (Fig. 1.4). The region is assigned to tropical savanna climate or tropical wet and dry climate, corresponding to the 'Aw'-climate of the 'Köppen climate classification' (Peel *et al.*, 2007), lies at the edge of the Atlantic Equatorial Coastal forest ecoregion (Olson *et al.*, 2001) and the forest type is classified as dry forest and thicket (White, 1983). However, the cold Benguela current in the Gulf of Guinea creates a thick, low-level, non-precipitating cloud layer during the dry season (Pendje & Baya ki, 1992). During this dry period, the inter-tropical convergence zone moves north,

yet a thick cloud cover results in high relative humidity as well as a decrease in temperature and solar irradiation, due to a lower sea temperature. Without this phenomena, the low precipitation values would not be favorable for the presence of the dense humid Mayombe forest (Couralet, 2010). Plants most probably do not experience extreme water stress during the dry period (Lubini, 1997; Couralet *et al.*, 2010). Therefore, the Luki reserve can be classified as tropical semi-evergreen forest of the Guineo-Congolean forest domain that consists of a mixture of evergreen and deciduous species in the upper-stratum and mostly evergreen species in the understory, with the hilly landscape causing moist green valleys and drier tops (Lubini, 1997). Within the Luki Reserve, the Nkulapark is a unique forest monitoring park established in 1947 by L. Toussaint, which monitored tree growth during 10 years, as well as phenology (Couralet *et al.*, 2013).

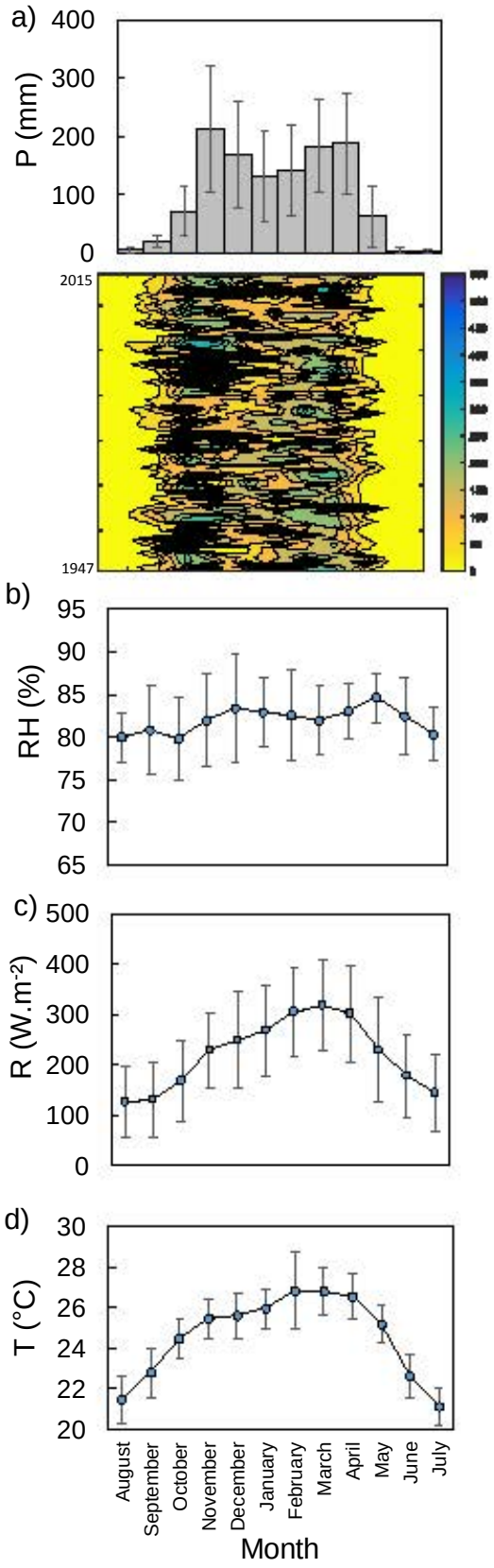

Figure 1.4 General weather pattern in the Luki reserve, with a) monthly precipitation values 1947-2015 showing the varying interpluvial and the dry season, b) relative humidity, c) solar irradiance and d) temperature (INERA, 2015). Error bands 1 standard error .

The original contribution of this PhD thesis is the exploration of the xylem archive of tropical trees, by assessing patterns of wood traits, in function of tree performance, as continuous variables rather than the discrete approach of counting and matching tree rings. Several research questions are at hand in this respect. Can we unlock the driving forces of wood traits? How can spatial patterns be converted to the time domain? Is a fast method available for measuring these traits and if not, can such a method be developed? What is the variability in wood traits between and within species in a tropical forest, and how does this affect tree performance? To answer these questions trees were live monitored in the field, phenology and wood traits were measured. State-of-the-art techniques were explored for wood trait analysis and the unique availability of a long-term dataset allowed to assess wood traits in function of long term tree growth, complementing, supporting and enhancing conventional tree ring analysis.

Following objectives were specified to achieve this contribution:

(i) Analyse intra-annual wood traits and fix them on a time axis through assessment of phenology of the axial and lateral meristems
(ii) Explore X-ray densitometry as a suitable method to describe intra-annual wood traits
(iii) Develop a toolchain to treat large sets of tropical tree increment cores in a fast and efficient way, and propose a method to enhance tree ring dating exploiting these intra-annual wood traits
(iv) Apply the above techniques on multiple species from different functional groups, from the seasonal scale to the multi-decadal scale

This dissertation is divided in seven chapters (Fig. 1.5) to achieve these research objectives.

Chapters 2 to 4 elaborate on the tree response of *Terminalia superba*, a widespread pioneer tropical tree species in the Mayombe. De Ridder *et al.* (2013) have assessed the climate-growth relationship by use of dendroclimatology on tree ring widths, yet there was a knowledge gap on seasonality of intra-annual traits.

Chapter 2 focusses on four selected *Terminalia superba* trees, where two years of tree monitoring of stem diameter variations, leaf and cambial phenology, are related to wood cellulose stable isotopes ($\delta^{18}O$, $\delta^{13}C$) and quantitative wood anatomy (vessels, parenchyma and fibres) of the two formed rings. Chapter 3 proves the relation between

QWA and the intra-annual density profile, based on X-ray CT scanning. Chapter 4 reports on the development of software for high-throughput processing of tropical (and temperate) tree cores in order to obtain density profiles that serve for tree-ring analysis, and a new method is proposed to assist the cross-dating process based on intra-annual density-based pattern matching.

Chapters 5 to 7 build further on the work of Couralet (2010) concerning phenology and wood formation of different functional groups in the Mayombe forest, and assesses wood formation from intra-annual to multi-decadal scale.

Chapter 5 studies monthly cambial activity for multiple understory species via cambial marking. Finally, Chapter 6 applies the X-ray CT toolchain to a core dataset of trees from a long-term forest monitoring experiment, which contains cambial markings of 66 years old and allows assessing long term forest growth. Chapter 7 discusses tree ring phenomena that occurred throughout the chapters, which are generally seen as a bottleneck for tropical tree ring analysis, but could provide potential opportunities in xylem trait research.

In Conclusions, a general discussion is given, followed by recommendations for future research.

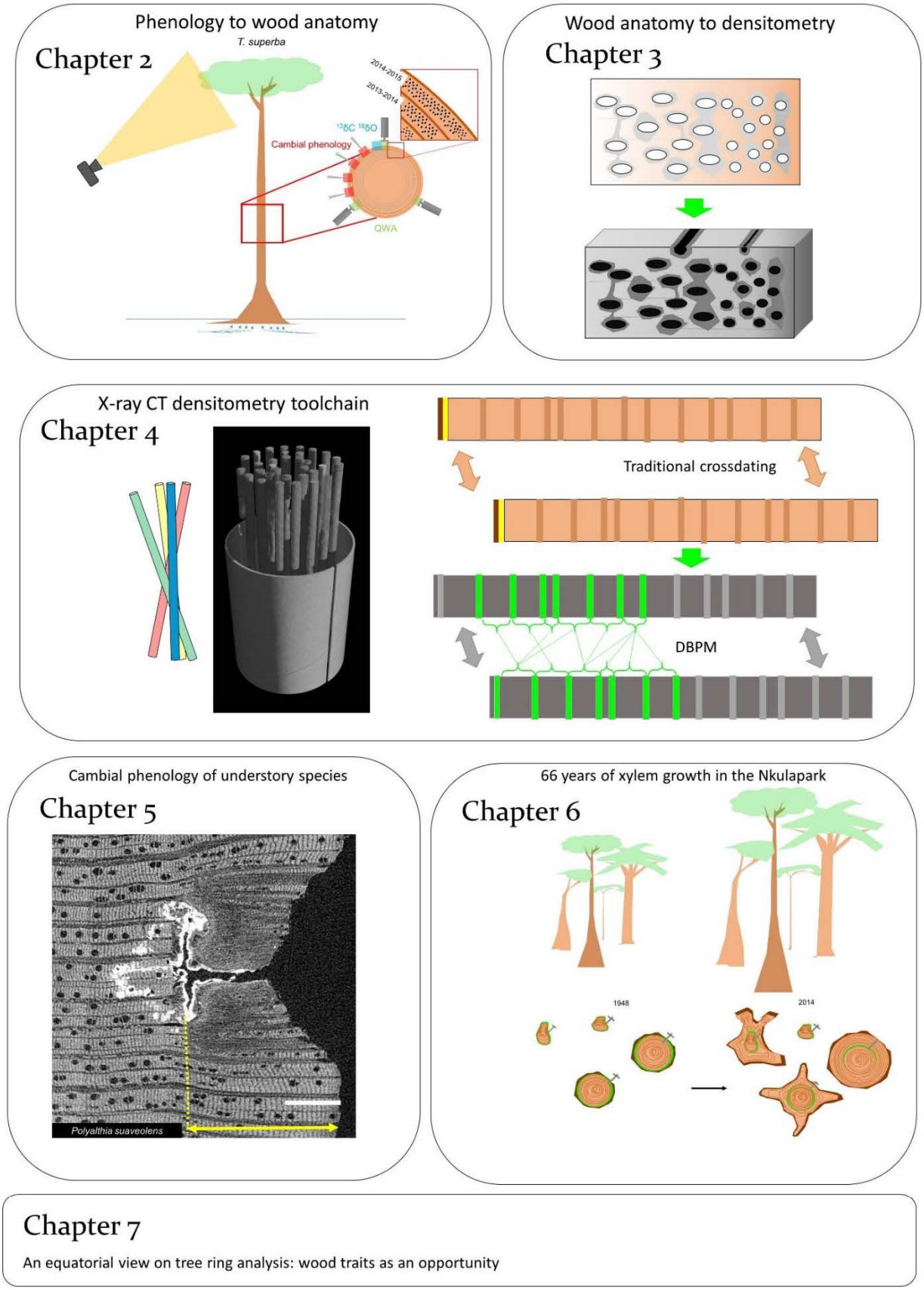

Figure 1.5 Thesis outline

2

Traces of leaf and cambial phenology in intra-annual wood traits

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Abstract

Xylem traits from pith to bark are increasingly being explored to map tree performance in the past. In tropical trees, linking xylem structure to the past is challenging and depends on a thorough assessment of leaf and cambial phenology. Here we studied phenology to determine the timeframe of radial growth in order to position the spatially determined wood anatomical variables and wood cellulose stable isotope composition on a seasonal time axis for *T. superba*. During two growing seasons, leaf phenology and cambial phenology were assessed to disentangle the response of four mature trees from the Central African Mayombe forest. The resulting rings were subjected to intra-annual wood cellulose $\delta^{13}C/\delta^{18}O$ analysis, vessel and parenchyma area measurements. Leaf onset differences were observed between trees and were constant between years, irrespective of rainfall onset, resulting in differing xylem onsets. This results in tree-specific isotope composition and wood anatomical traits. Increasing droughts could impact *T. superba*, given that transpiration can start before actual rainfall. Differences in cambial phenology result in tree-specific timeframes of growth, and the resulting intra-annual traits should be assessed on a continuous time axis, rather than on a distance axis within the tree ring.

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Preface Chapter 2

T. superba trees from the Mayombe have a pronounced ring formation (De Ridder et al., 2011), but many samples are rejected due to lack of cross-dating. One reason might be the variation in leaf phenology, even of trees in the same stand (Fig. 2.1 a), and specifically in the Luki reserve variation is shown in the number of T. superba trees shedding their leaves in the Nkula catchment (Fig.2.1 b), another reason is that tree ring boundary alone might not be sufficient to disentangle the subtle response of tropical trees to climate, so that we need to consider intra-annual wood traits. This chapter monitors leaf and cambial phenology to a) assess the variation between these two meristems and b) whether we can enhance tropical tree response via intra-annual wood traits, such as wood cellulose stable isotopes and wood anatomical traits.

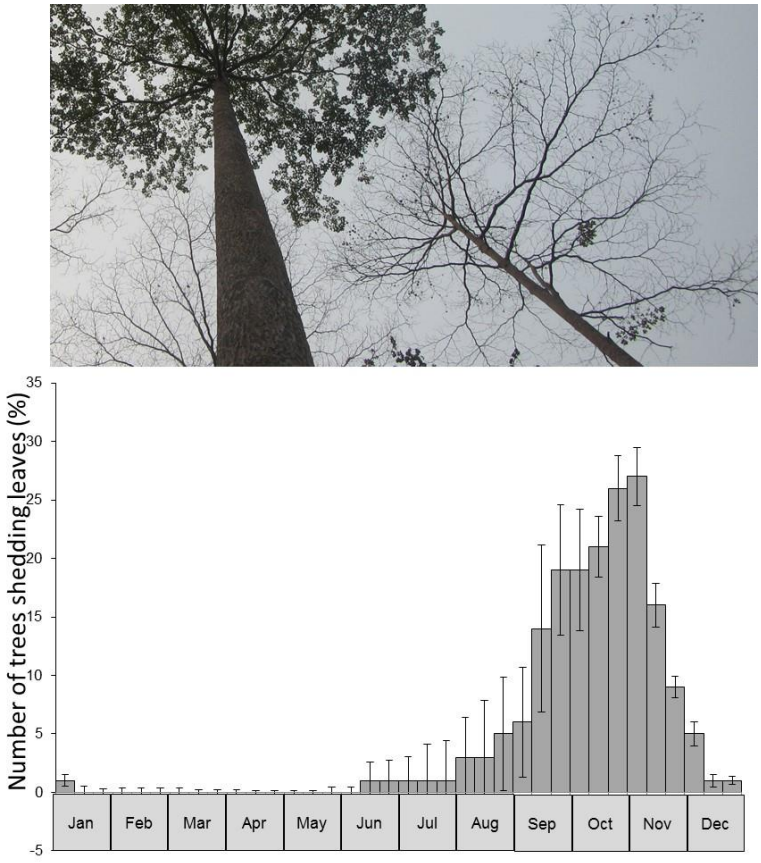

Figure 2.1: Terminalia superba in the Luki reserve, with varying leaf phenology between trees. (a) Illustration of two adjacent individuals in the dry season (18/08/2013), one with developed leaves, whilst the other tree has shed all his leaves, and (b) fraction of T. superba trees that are shedding their leaves per month during the period 1948-1957, based on 184 trees from the digitized database of Couralet et al., (2013). Error bands show 1 standard error.

2.1 Introduction

Future climate changes will affect plant growth (Reyer *et al.*, 2013; Cavaleri *et al.*, 2015) and are expected to push tropical ecosystems into a novel, unprecedented state (Holm *et al.*, 2017). Tropical forests are a major sink for carbon, and studying their response is essential to understand aforementioned future impact (Lewis *et al.*, 2009). The largest part of forest biomass consists of wood and bark (Beeckman, 2016) and studying radial growth is therefore essential and is all the more urgent in the tropics given the uncertainties of tree carbon gain and their specific vulnerability to drought (Zuidema *et al.*, 2013; Gebrekirstos *et al.*, 2014). Periodical repeated measurements of radial growth in the tropics (Lewis *et al.*, 2009; Brienen *et al.*, 2015; Popkin, 2015) range back to decades, whilst the analysis of series of yearly growth ring descriptors of wood structure (Worbes, 1989; Schöngart *et al.*, 2006) spans centuries of growth. In tropical trees traditional tree ring analysis is often problematic due to ring wedging (Worbes, 2002; Groenendijk *et al.*, 2014) or unclear ring boundaries (Tarelkin *et al.*, 2016).

Therefore, there is a clear need to include more traits than only tree ring width to derive environmental information from (Fonti *et al.*, 2010; Battipaglia *et al.*, 2014b; Sass-Klaassen, 2015). Often only used as a means for identification of tree ring boundaries in tree-ring analysis, wood traits carry underexplored intra-annual ecological information from the past (Fonti *et al.*, 2010), especially in tropical trees.

Establishing such high-resolution or intra-annual chronologies (Eckstein, 2004; Rossi & Deslauriers, 2007) of wood traits can include elemental (Poussart *et al.*, 2006; Cherubini *et al.*, 2013; Hietz *et al.*, 2015), wood stable isotope (Evans & Schrag, 2004; Helle & Schleser, 2004; Poussart *et al.*, 2004; Verheyden *et al.*, 2004; Fichtler *et al.*, 2010; Managave & Ramesh, 2012), and wood anatomical traits (Battipaglia *et al.*, 2010; Fonti *et al.*, 2010; von Arx *et al.*, 2016). They go beyond conventional tree-ring width analysis, and allow us to have a deeper understanding of the underlying mechanisms of xylem formation under environmental conditions.

Concentrations of stable isotopes of cellulose in leaves and wood ($\delta^{13}C$, $\delta^{18}O$) are of special interest in the tropics, as a proxy for physiological processes such as stomatal conductance, intrinsic water use efficiency (van der Sleen *et al.*, 2014) and source water use, as well as their use as a tree ring descriptor (Poussart *et al.*, 2006). Especially in the wood at the intra-annual level, they show clear links with environmental conditions such as monsoon rains (Schollaen *et al.*, 2014).

However, high-resolution intra-annual isotope studies contain several challenges of previous-year effects (Schleser *et al.*, 1999; Gessler *et al.*, 2014). Wood anatomy is also affected by previous years, (cf. autocorrelated growth Fritts, 1976), but is more likely to reflect the current environmental conditions, due to its close relation with cambial phenology (Fonti *et al.*, 2010).

Studies combining isotopes and wood anatomical traits linked to hydraulics such as vessel area, as such relating physiological processes to hydraulic properties, are therefore increasing in number (Battipaglia *et al.*, 2014a), but are lacking in tropical regions. Many wood anatomical traits are multifunctional (Beeckman, 2016) and non-conducting traits that have a mechanical function such as fibres (Moya & Tomazello-Filho, 2007; Dié *et al.*, 2012; Fichtler & Worbes, 2012; De Micco *et al.*, 2016) or storage function such as parenchyma (Olano *et al.*, 2013; Morris *et al.*, 2016) are increasingly being considered as potential proxies for tree response.

For a good understanding of growth patterns on a pith-to-bark gradient, there is a need to bridge carbon accumulation to seasonal mechanisms of wood formation (Zweifel *et al.*, 2006, Worbes *et al.*, 2013; Steppe *et al.*, 2015), given that there is a manifest relation between wood structure and tree performance. However, in tropical trees, exploring the xylem archive via tree ring analysis is problematic. The solution for these problems can be found by assessing what happens throughout the season, between the ring boundaries: a sound interpretation of growth-ring patterns of tropical trees, depends on understanding phenological rhythms (Enquist & Leffler, 2001) at the primary meristem level (leaf phenology) and at the secondary meristem level (cambial phenology).

Tropical tree response to climate change is typically studied via leaf phenology observations made on satellite images (Ryan *et al.*, 2016) or *in situ* field observations of leaf and flowering timings (Borchert *et al.*, 2005; Fu *et al.*, 2015). Periodicity and intensity of droughts depend from region to region (Worbes *et al.*, 2013) and tree species vary in response strategy to these droughts via phenology adaptation. Several tree types can be distinguished, ranging from evergreen to brevi-deciduous to deciduous (Borchert, 1994) that occur in the same forest (Borchert *et al.*, 2005) and can be ascribed to functional types (Couralet *et al.*, 2013; Wolfe & Kursar, 2015). In Central Africa, a shift in functional types from evergreen understory and canopy to light demanding deciduous species is caused by increased drought (Fauset *et al.*, 2012). Variability at the individual level in phenology is important as well (Visser *et al.*, 2010; Forrest & Miller-Rushing, 2010). It is well known that the degree and synchronicity of leaf and/or flowering phenology is an indicator of the environment across species (Borchert *et al.*, 2005) and between trees of the same species

(Heinrich & Banks, 2006), that will, depending on the intensity of climatic conditions in the tropics, control cambial phenology (Borchert, 1999; Lisi *et al.*, 2008; Yanez-Espinoza *et al.*, 2010).

Cambial phenology is more difficult to measure, but is archived in the secondary xylem (Beeckman, 2016) via radial growth. Therefore, cambial phenology might be even more important to monitor as it can be assessed retrospectively and can directly be linked to the xylem archive. Monitoring cambial phenology at weekly to monthly intervals (Cuny *et al.*, 2016) provides a mechanistic understanding of seasonal wood formation and carbon accumulation. Monitoring Stem Diameter Variations (SDV) is essential for these cambial dynamics, revealing (i) physiological responses through stem water relations (Zweifel *et al.*, 2001; Steppe *et al.*, 2006) as well as (ii) seasonal growth (Deslauriers *et al.*, 2003; Steppe *et al.*, 2006), making it a versatile research tool (De Swaef *et al.*, 2015). SDV reflect swelling and shrinking due to water status, with xylem increment as only one component contributing to this pattern (Mäkinen *et al.*, 2008). Therefore, cambial sampling (Mariaux, 1969; Amobi, 1973; Rossi *et al.*, 2003; Seo *et al.*, 2007; Gricar *et al.*, 2011; Trouet *et al.*, 2012) is key as a complementary tool (Steppe *et al.*, 2015) since it directly documents irreversible xylem growth.

Dating intra-annual wood traits on a seasonal time axis is essential to interpret the physiological mechanisms (Bouriaud *et al.*, 2005) to drought. Thus, in the case of tropical trees, it is vital to convert intra-annual traits measured in distance units, to actual time units, so that intra-annual features can be directly ascribed to “live measured” phenological events during the season. Moreover, the exact period of tree ring formation within one growing season varies with age and site (Philipson, 1971; Rossi *et al.*, 2008).

Given the challenging task of assessing climate sensitivity in the tropics, there is a clear need for an integrated approach of improving tropical radial growth estimates, with the incorporation of intra-annual traits that are coupled to leaf and cambial phenology.

This chapter reports on such a multidisciplinary bottom-up study of intra-annual ring formation of *Terminalia superba*, which has a pronounced leaf phenology (Couralet *et al.*, 2013), a demonstrated tree-ring analysis potential and a considerable intra-annual anatomical variation (De Ridder *et al.*, 2011, 2013; De Mil *et al.*, 2016), but knowledge on the mechanisms of the latter, remains elusive. The concept of radial growth in tropical trees is assessed via a framework that encompasses weather conditions and the effect on tree water relations, leaf and cambial phenology, and the resulting xylem archive with its isotopic and wood anatomical traits (Fig. 2.2).

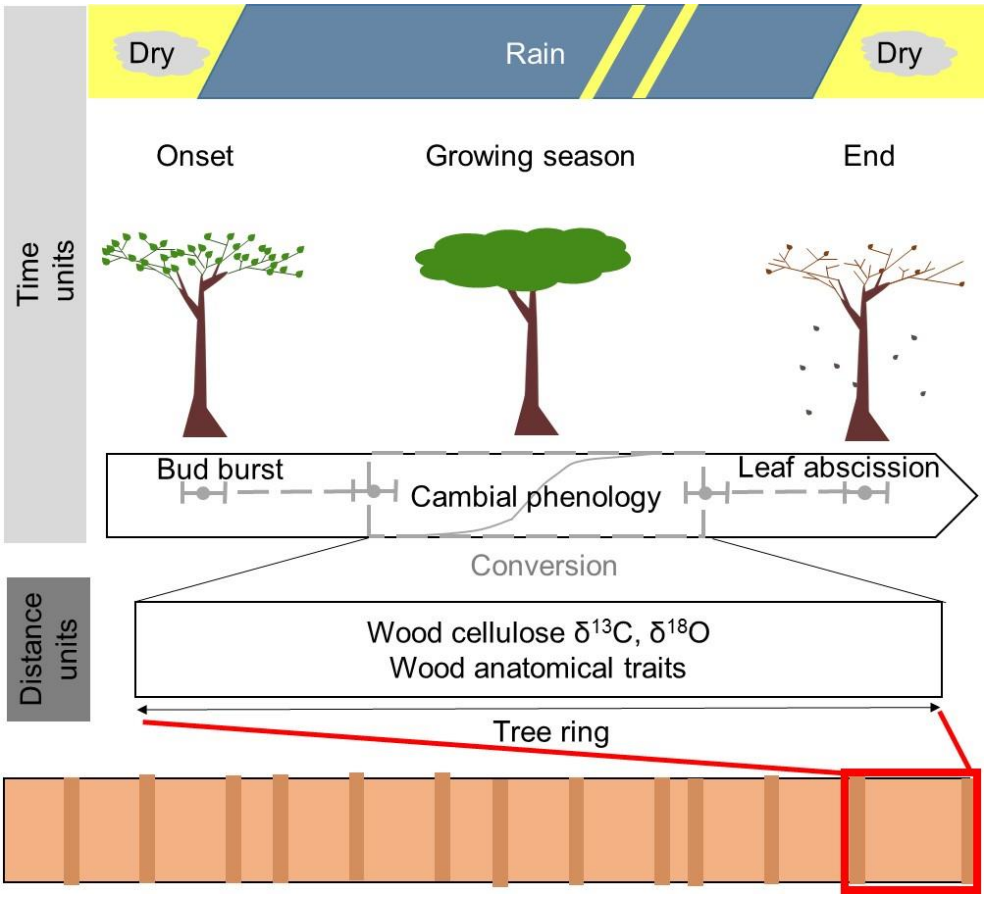

Figure 2.2 Framework for a multidisciplinary approach on the tropical deciduous species Terminalia superba, relating meteorological variables, leaf phenology and their synchronicity with cambial phenology at the stem base. The latter provides the conversion between xylem traits that are measured spatially on a tree ring (cellulose isotopes and vessel, parenchyma area and fibre proportion), and the phenology and climatic variables measured in function of time.

In this respect, we hypothesize that timing of leaf and cambial phenology are determining the pattern of wood cellulose stable isotope $\delta^{13}C$ and $\delta^{18}O$ and wood anatomical traits within a tropical tree species, and that they can result in low cross-dating quality of classic tree-ring analysis.

We combined (i) in-field monitoring – cambial wounding, phenology cameras, point dendrometers and local weather stations – during two growing seasons followed by (ii) lab measurements – isotope and wood anatomical analysis – of the resulting growth rings of the corresponding seasons.

2.2 Material and Methods

Site description

The Luki reserve (5° 37.31' S, 13° 5.90' E) lies at the southern edge of the Mayombe, which is an Atlantic Coastal forest ranging from Gabon to western DR Congo (Fig. 2.3a). The rainy season (average 1188 ± 245 mm) lasts from October to May, yet the dry season is less pronounced due to thick cloud cover, high relative humidity and occasional occult precipitation (Lubini, 1997).

Tree monitoring: Leaf phenology and meteorological observations

Terminalia superba is a widespread long-lived pioneer and one of the tallest trees in the Mayombe forest. Its distribution area ranges from Sierra Leone, along the West African coastal line up to the northern boundary of Angola, thus the Luki reserve is at the edge of its distribution limit. Four trees from different local sites were selected and monitored from August 2013 to December 2015, covering three season onsets and two full growing seasons. The crowding index as a measure for neighboring influence of trees (Michelot *et al.*, 2011), was calculated as well (Table 2.1, Supplementary Material Fig. S2.1).

Table 2.1: General characteristics of the sampled trees

Tree	Height (m)	DBH (m)	Est. Tree age (yr)	Proj. crown area (m²)	Crowding index (-)	Site info
T1	35	0.47	35	142.5	2.23	Young secondary forest
T2	45	0.69	70	223.2	1.70	Old secondary forest
T3	39	0.65	85	213.6	2.10	Old secondary forest
T4	31	0.83	47	530.0	1.36	Free standing tree

Three point dendrometers (EcoMatik, Germany) were installed on each tree to account for circumferential variation (Fig. 2.3b). The dendrometers were installed at 4-5 m height, to reduce the influence of large buttresses. Stem Diameter Variations (SDV) were logged every 30 minutes using ONSETComp HOBOloggers (Onset, USA). Time lapse cameras (Wingscapes, USA) (Sonnentag *et al.*, 2012) were installed on adjacent trees and directed towards the studied trees for leaf phenology monitoring: 2-3 pictures/day were taken for two consecutive growing seasons. The large buds and composed leaves of *T. superba* are clearly visible on the images. Time lapse images were visually analysed and leaf events were classified into defined phenology phases, generally adapted from Derory *et al.* (2006). Daily precipitation was recorded at the INERA meteorological station of the Luki reserve (Fig. 2.3a). Rain onset was defined based on a conservative threshold for trees:

10 days with a cumulative sum of 25 mm, followed by 20 days of > 20 mm (Ryan *et al.*, 2016). The two monitored years contain no exceptional drought compared to the 1947-2015 averages from the Luki INERA station (Fig. 2.3b).

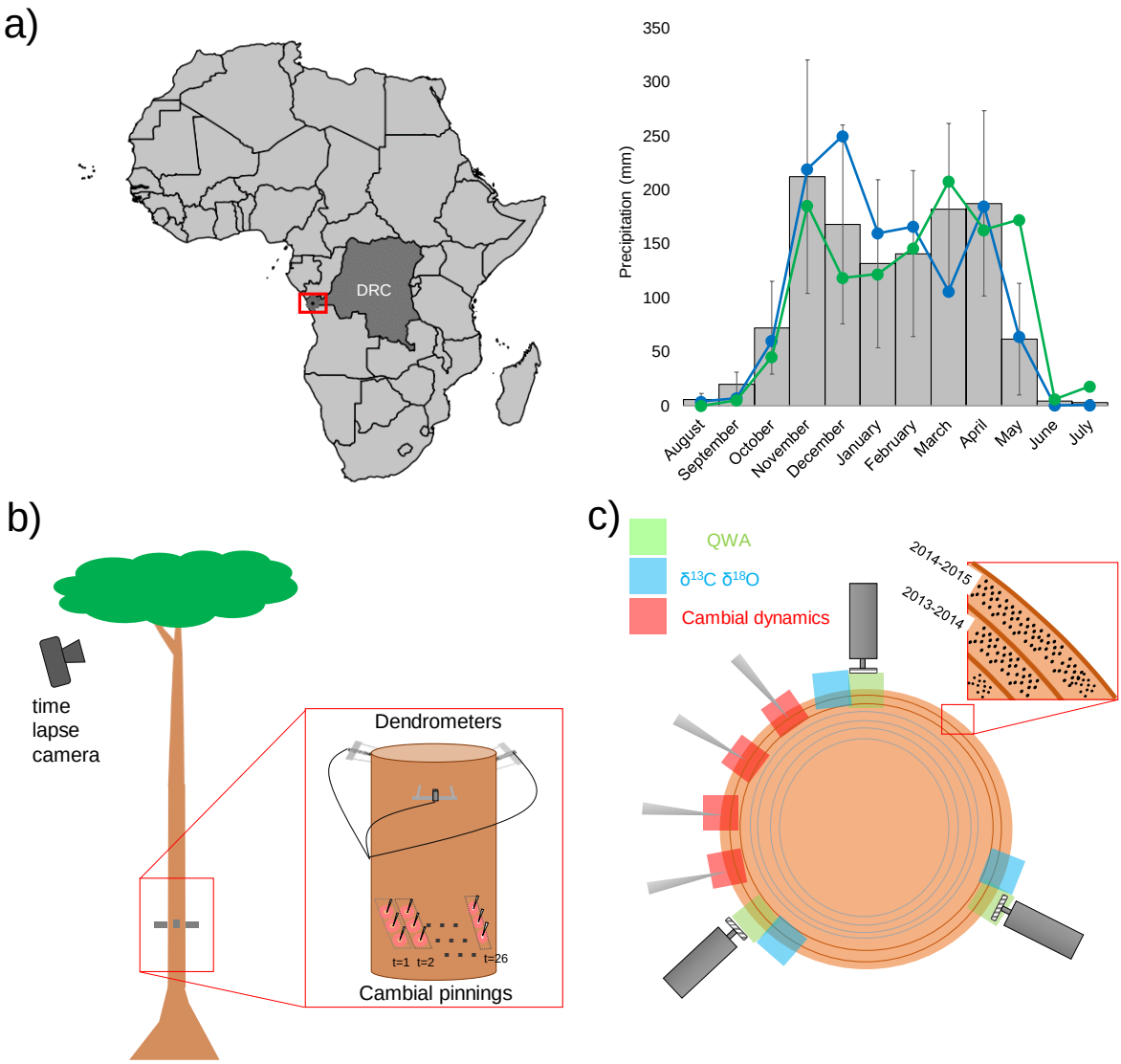

Figure 2.3 Study area and experimental setup. (a) Mayombe study site (DR Congo) for monitoring Terminalia superba trees, with precipitation averages (INERA Luki), precipitation of the 2013-2014 and 2014-2015 seasons are indicated with blue and green dots, respectively. (b) Monitoring of leaf phenology with time lapse cameras, stem diameter variations (SDV) with point dendrometers and xylem growth using monthly cambial pinnings. (c) Sampled stem disks showing dendrometers each at 120° on the circumference; pinning samples taken for cambial dynamics, quantitative wood anatomy (QWA) and samples taken for cellulose carbon $\delta^{13}C$ and oxygen $\delta^{18}O$ stable isotopes are also indicated.

Cambial pinning

Monthly cambial pinnings were performed at a height between 0.4 m and 1 m below the point dendrometers in a staggered pattern around the circumference (Fig. 2.3b). Stem disks were harvested in December 2015 and cylindrical samples containing the pinnings (Fig. 2.3c) were extracted from the disks and surfaced above or below the pinning spot. Intra-annual growth was measured using a stereo microscope and a Lintab measuring stage in combination with TsapWin software (Rinntech, Germany). Relative growth (RG) was calculated to obtain seasonal xylem growth curves, given that variability of absolute growth along the circumference can be too high to construct a growth curve that is typical of the individual (Seo *et al.*, 2007; De Mil *et al.*, 2017).

Vessel area, parenchyma area and fibre wall/lumina measurements

At the exact position of the point dendrometers, tree rings of the two monitored growth seasons were cut from the stem disks, carefully sectioned, stained with safranin and alcian blue, and imaged (StreamMotion, Olympus, Japan) on a scanning stage (SCAN 100 x 100, Märzhäuser Wetzlar, Germany) with a camera (UC30, Olympus) mounted on a microscope (BX60, Olympus, magnification 5X). Automated stitching of the images enabled to visualize both growth rings in a single image. Vessel frequency, vessel area were measured in ImageJ (Schneider *et al.*, 2012) via manual selection using the magic wand tool, whereas parenchyma was manually measured. Fibre lumina and wall thickness were measured on X20 magnification images via a method based on pattern recognition (for details see Section 3.2.1).

Cellulose stable isotope analysis

Adjacent to the point dendrometer positions, the disks were sampled for intra-annual isotope analysis ($\delta^{13}C$ and $\delta^{18}O$). A Lintab measuring stage with a stereomicroscope (SZX 12, Olympus) was used to cut the blocks (1 cm x width of the tree rings) into equal radial slices with an approximate thickness of 0.3 mm. Cellulose extraction of these radial slices was performed at the ISOFYS Lab. $\delta^{13}C$ was measured using an Automated Nitrogen Carbon Analyzer – Solid and Liquids (ANCA-SL, SerCon, UK) interfaced with an Isotope Ratio Mass spectrometer (IRMS) (20-20, SerCon, UK), whereas the same slices were used for $\delta^{18}O$ determination using a Thermal Conversion Elemental Analyzer (Sercon, UK) interfaced with an IRMS (20-20, SerCon, UK). The $\delta^{13}C$ and $\delta^{18}O$ isotope values are expressed against the Vienna PeeDee Belemnite and the Vienna Standard Mean Ocean Water, respectively.

Both isotope series, as well as the wood anatomical traits were further processed in Matlab r2016b.

Distance units to time units: combining stem diameter variations and cambial pinning

Cambial pinning is used to separate xylem growth from total radial increment as it can be read from the SDV curve (Fig. 2.4). The date of onset of xylem growth is evidenced from the cambial pinning experiment and finetuned via the SDV series. This date can then be considered as starting point of xylem formation. Saturated SDV series, i.e. those cases where xylem growth exceeded the measurement range of the dendrometers due to fast growing rates, were corrected and completed by interpolation based on (i) the total tree ring width and (ii) cessation dates from the other radii of the same tree without sensor saturation. All series were median filtered.

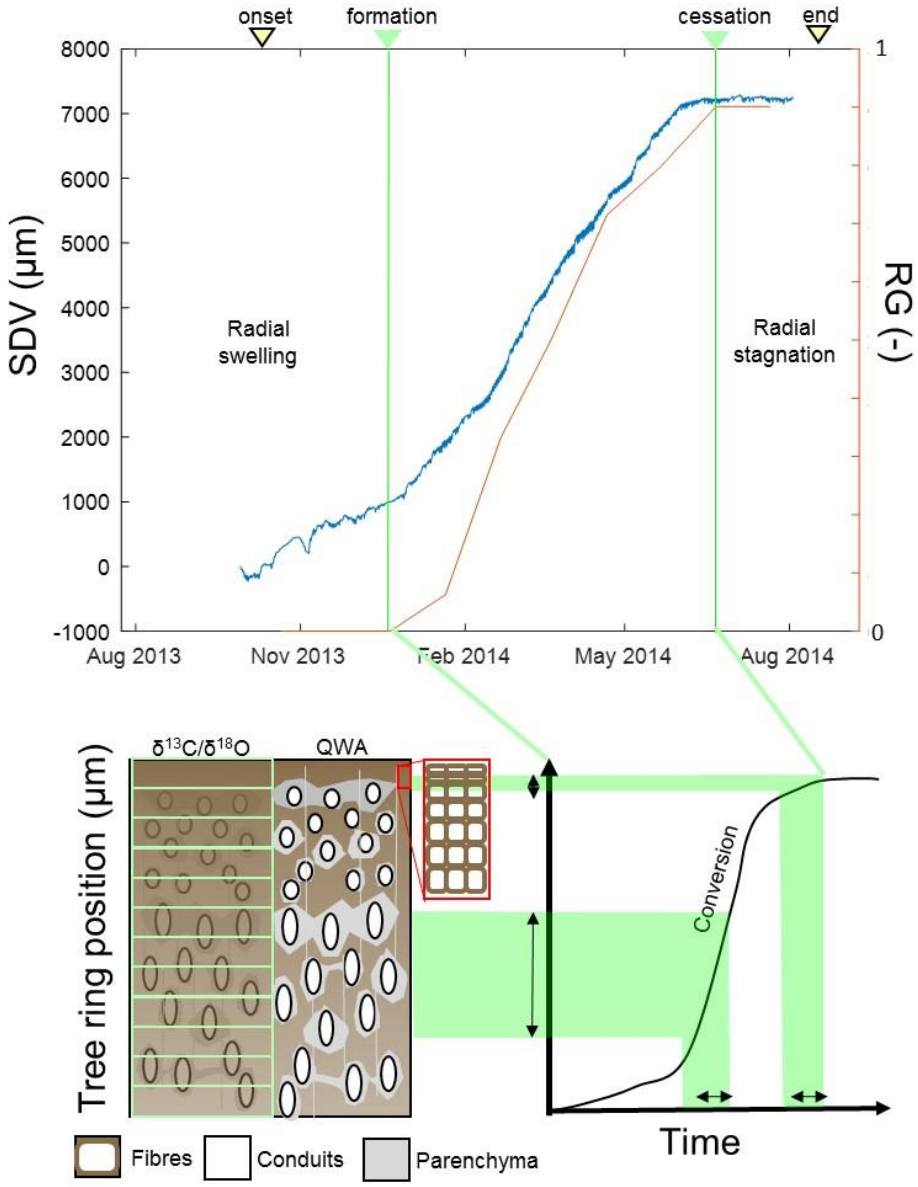

Figure 2.4 Relative growth (RG, brown line) obtained by cambial pinning separates xylem growth from total radial increment based on daily stem variations (SDV, blue curve) to determine the xylem growing season as well as to establish a time/space conversion for vessel traits as well as wood cellulose $\delta^{13}C$ and $\delta^{18}O$.

A smoothed (Savitsky-Golay) radial curve, eliminating diel variations, was then fitted to the SDV and used to convert wood variables to the time domain.

2.3 Results

2.3.1 Leaf phenology and SDV: onset and end of the growing season

Bud burst

Leaf phenology is illustrated in Figure 2.5a for T1. Defined leaf phenology phases are 1) old leaves present, 2) leafless, 3) bud swelling, 4) bud burst, 5) leaf development and 6) full leaf stage. Figure 2.5b links these phenology phases to SDV during the three consecutive season onsets. Tree T2 and T3 showed an identical leaf phenology. The three radii show a similar pattern, therefore, only one radius per tree is displayed. Figure 2.5b includes rainfall data for the end of the dry season and the onset of the next rain season. Between the trees considerable variation is present in leafing timing up to 45 days (Fig. 2.5b Table 2.2). For all three monitored onsets, flushing occurs before (T1,T4) as well as after the first major rains (T2, T3), except for the 2015 rain onset, where all trees flush before the onset of rain, starting later than the previous seasons. Tree water status, indirectly captured via SDV, fluctuated with precipitation: trees that flushed before the rain onset, showed a strong decrease in tree water status, and when the first rains occurred, the stem radius increased. For trees that flushed after the rain onset, there was no decrease. Although tree water status as well as precipitation varied between the 2013, 2014 and 2015 onsets, leaf phenology timing remains rather constant each year. The amplitude of the daily SDV increases once the tree flushes.

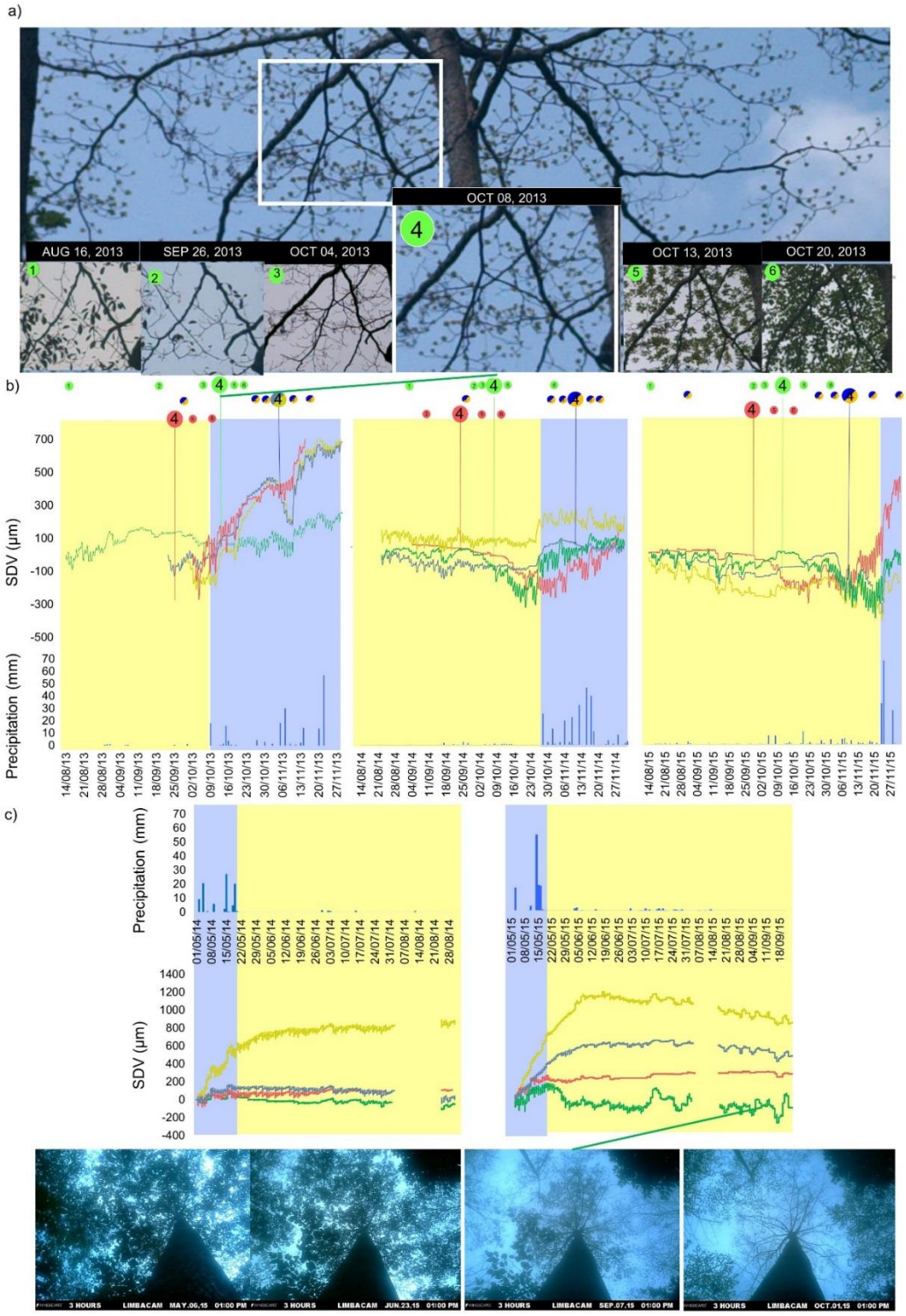

Figure 2.5 Relation between leaf phenology, precipitation and Tree Water Status (TWS). (a) Leaf onset monitoring of a Terminalia superba tree (T1), each tree is represented by a colour (T1: green, T2: blue, T3: yellow, T4: red) and numbers correspond to 1) old leaves present, 2) leafless, 3) swelling, 4) bud burst, 5) leaf development and 6) full leaf stage, during the onset of the rainy season (blue shaded), together with (b) stem diameter variations (indirect measure for tree water status TWS when radial stem growth is eliminated). (b) End of rainy season and associated leaf fall behaviour and TWS changes of T1. A gradual leaf fall is observed, far in the dry season (yellow shaded).

Leaf abscission

The end of the season, i.e. leaf senescence and abscission (cf. Fig. 2.2), is not sharply defined for *T. superba*. Despite the lack of rainfall at the end of the season, all trees maintain a large fraction of their leaves far in the dry season (Fig. 2.5c) and even after the next rainfall onset. The earlier the flushing in the previous year, the earlier the leaf shedding. The fully deciduous period is short for all trees, ranging from 5 days up to 25 days. During this period, the SDV are slightly decreasing or remain stable (Fig. 2.5c). For T2 and T3, old leaves are still present on the tree, while new leaves are already flushing (data not shown).

2.3.2 Intra-annual xylem growth: growing season length

Xylem growth, calculated according to the procedure described in Fig. 2.4, is illustrated in Fig. 2.6 for all radii of the four trees during two growing seasons. The original RG series are in Supplemenary Material Fig. S 2.2.

Xylem growth for all trees starts between November and January and lags leaf onset, ranging from 39 to 67 days, and exceptionally 80 days for T1 in 2014 (Table 2.2). The trees thus start xylem formation well in the wet season. Similar to the timing of leaf flushing and shedding, when trees start growing earlier, growth cessation starts earlier as well and these differences persist between the monitored trees and throughout the monitored seasons. On wet sections just taken after felling of the trees in December 2015, (non-lignified) wood formation could be observed for the early-onset T4 tree, whereas all the other trees did not show cambial activity yet, which confirms these observed differences (Supplementary Material Fig. S2.3). Between radii, the onset is identical (Fig. 2.6b).

Total xylem growing season length is about 6 months and is rather constant except for tree T1 that shows a short season of 4 months. During the xylem growing season, a large variability in growth rate is observed between trees. Therefore, some trees attain the same ring width, despite having different xylem growth onsets. Contrary to the consistent pattern between radii of short-term SDV during flushing, this is not true for the irreversible seasonal growth (Fig. 2.6): radial growth starts at the same time, but sometimes large differences in final increment can be found, causing irregular tree ring width along the circumference. On some radii an abrupt radial growth cessation is noted.

For trees T1 and T3 there was no ring formed in the growing season 2013-2014. As such, the little net growth observed for some radii of these trees in 2013-2014 in the SDV series

is due to water status, as well as little wound-induced xylem formation of the dendrometer support, as confirmed by visual control on stem disks (Supplementary Material Fig. S2.4). In 2014-2015, these trees formed a ring on all radii.

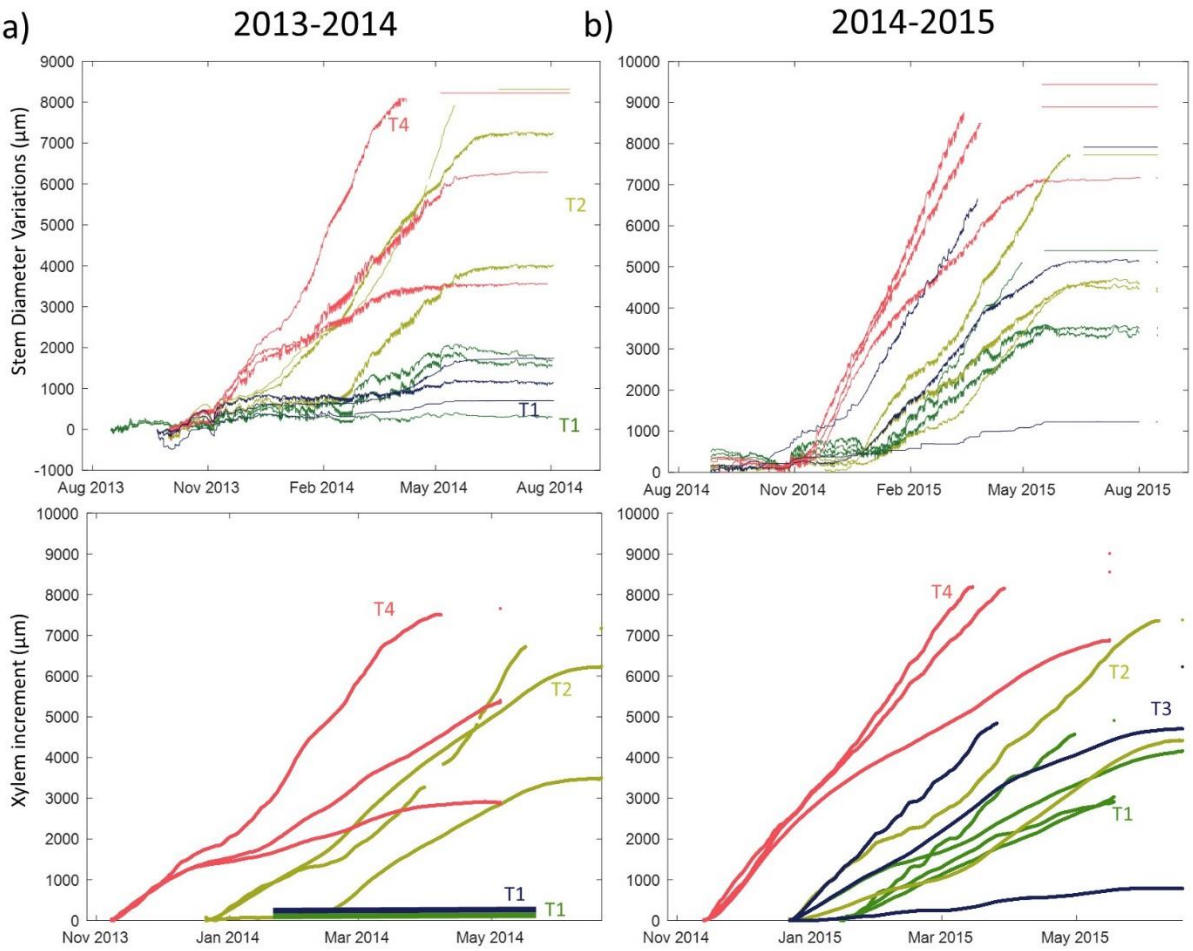

Figure 2.6 SDV profiles. a) raw SDV profiles and b) xylem growth derived from SDV profiles via cambial pinning for the season 2013-2014 and 2014-2015. Each colour represents a tree (3 radii per tree).

Table 2.2 Overview of leaf (Bud burst +-2 days) and cambial phenology (Onset & end Xy, +- 5 days) timing (DOY), the lag between them, and length of xylem growing season (days).

	2013			2014		2014			2015		2015		
Sample	Bud burst	Onset Xy	lag (d)	end Xy	Xy season (d)	Bud burst	Onset Xy	lag (d)	end Xy	Xy season (d)	Bud burst	Onset Xy	lag (d)
T1	280	-	-	-	-	276	14	80	138	124	280	-	-
T2	302	-	-	-	-	313	356	43	169	178	307	-	-
T3	302	355	53	171	181	313	356	43	169	178	307	-	-
T4	273	312	39	125	178	268	317	49	137	185	263	330	67

2.3.3 Intra-annual wood traits

The $\delta^{13}C$ profiles are illustrated in Fig. 2.7a in distance units and in Fig. 2.7b in time units. All profiles are positioned on a time axis for correct interpretation. When positioned on a distance axis, opposing trends would be wrongfully compared. The isotope pattern of the different trees does not entirely overlap since xylem onsets and cessations are different.

Generally speaking, $\delta^{13}C$ increases at the beginning of the growing season, culminates and starts decreasing around February-March (Fig. 2.7b) with a minimum in June, after which growth cessation occurs. The seasonal $\delta^{13}C$ interval ranges between -27.11 ‰ and -23.28 ‰ and the maximum intra-annual range within one ring is 3.42 ‰, the minimum range 1.76 ‰.

The $\delta^{18}O$ values do not show a clear trend for both years in all trees (Fig. 2.7c), and the interval ranges between 22.59 ‰ and 35.30 ‰ and the largest intra-annual range within one ring is 9.24 ‰, and minimum range is 6.35 ‰.

The vessel area (Fig. 2.8) shows a minimum around February-March for some rings, resulting in two vessel peaks. Not all trees show these two peaks, as later onset trees only show one peak.

Due to shifts, it is seen that vessels in one tree (T4) are forming 42 days earlier than the other tree (T2). For parenchyma (Fig. 2.9) a similar pattern is seen, albeit less clear. Both parenchyma and vessels gradually decrease at the end of the tree ring boundary for all trees. All trees show a fibre wall fraction increase towards the end, with intra-annual peaks (Fig. 2.10). Rings on the radii of T1 and T3 were missing in 2013-2014 and some rings in 2014-2015 were too small to be measured, and thus, not considered here.

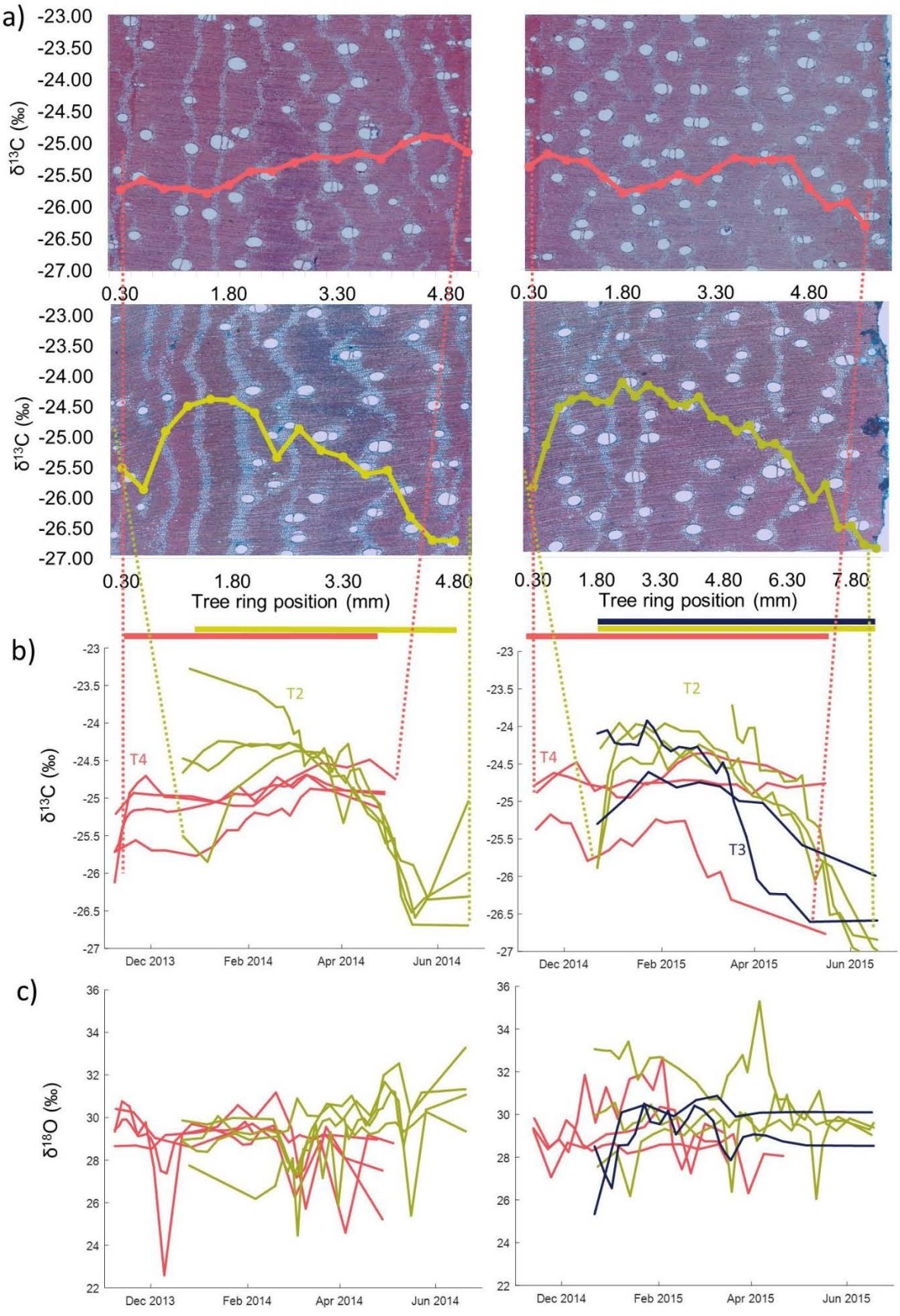

Figure 2.7 a) Cellulose $\delta^{13}C$ pattern for tree T2 and T4 in 2013-2014 and for T1,T2,T3,T4 in 2014-2015 plotted on a distance axis, b) plotted on a time axis b) $\delta^{18}O$ pattern plotted on a time axis.

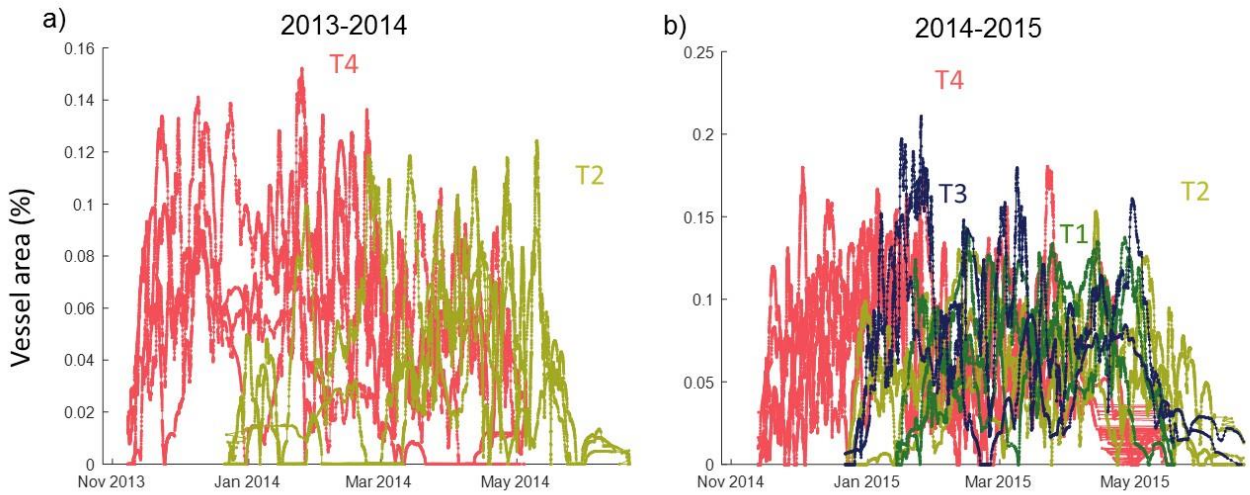

Figure 2.8 Time series of vessel area for a) T1, b) T2, c) T3 and d) T4.

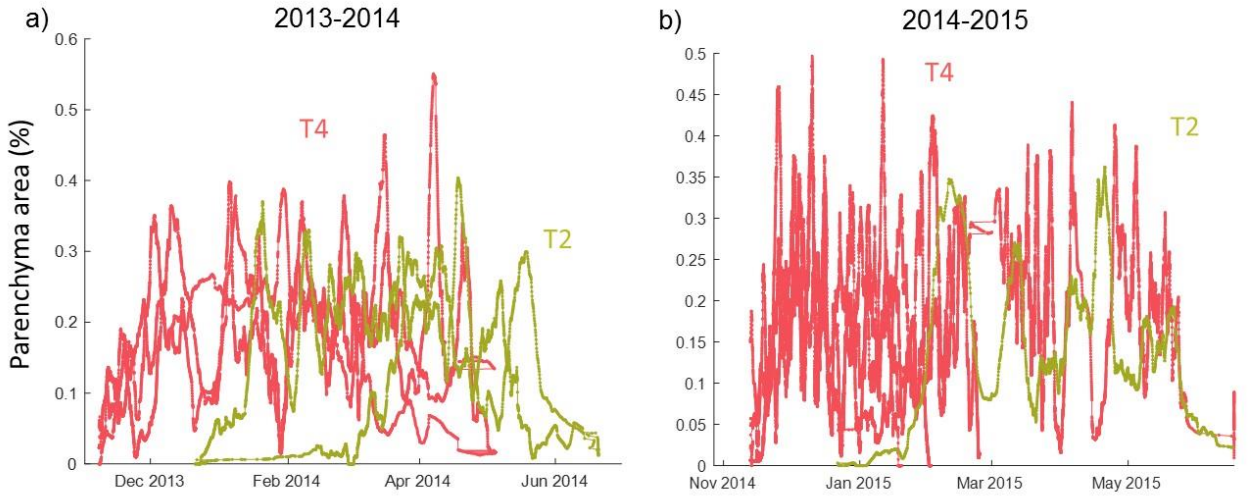

Figure 2.9 Time series of parenchyma area for a) T2 and b) T4.

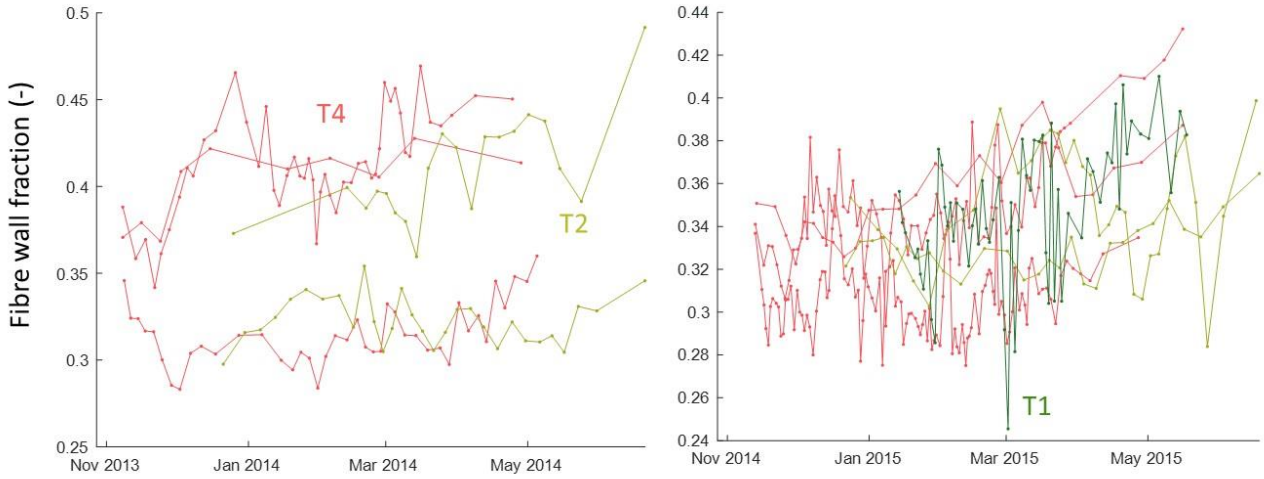

Figure 2.10 Fibre wall fraction, expressed as fibre wall size divided by the sum of wall size and lumina at that position. a) T2 and b) T4 during the two seasons.

2.4 Discussion

2.4.1 Leaf onset before rain onset

The pre-rain green-up known in Southern Africa (Ryan *et al.*, 2016) is shown here for *T. superba,* with little differences in timing between the seasons, but large differences between individuals. Despite general species-specific phenology pattern in the Mayombe, individual differences between dates of leaf onsets in *T. superba* (Fig. 2.5a) have also been reported (Mariaux, 1969; Couralet *et al.*, 2013), describing a broad leaf onset distribution (Fig. 2.1b). The timing of the event of leafing seems to be determined in each individual whether or not due to intrinsic or site conditions, irrespective of the precipitation onset: the 2013, 2014 and 2015 leaf onsets appear similar each year (Table 2.2), despite that the rain onsets differed considerably. Other drivers such as insolation or photoperiod might thus be of influence as well (Borchert *et al.*, 2015), yet it does not explain the individual differences.

SDV series can be considered a proxy for TWS when radial growth is eliminated from the measured signal (Zweifel *et al.*, 2001; Steppe *et al.*, 2006), especially for species with a relatively thick bark (Borchert, 1994) such as *T. superba*. Some trees in our dataset release water during flushing, seen as a decrease in the SDV measurements (Fig. 2.4b) when still in the dry season, supporting the condition of leaf flushing through available stem water content in deciduous species with low wood density (Wolfe & Kursar, 2015), such as *T. superba* (De Ridder *et al.*, 2011).

Thus, intrinsic factors could have an influence rather than a direct effect of precipitation: leaf flushing could merely be a consequence of leaf shedding and the resulting tree water status changes (Borchert, 1999). *T. superba* can then be considered a brevi-deciduous tree according to Borchert (1999) (where trees tend to be leafless only in a short period, and flushing follows leaf shedding), and stored stem water causes some trees to flush before the onset of the rainy season (Borchert, 1994).

Some stem shrinkage is observed in this study as well as a gradual leaf shedding up to the short period of deciduousness (Fig. 2.5c). In the specific case of the Mayombe, water loss during the dry season could also be limited due to reduced vapour pressure deficit and evapotranspiration (Supplementary Material Fig. S2.5), as well as occult precipitation. Thus, the dry season does not completely concur with the leafless season.

2.4.2 Lag leaf and cambial phenology and differences in onset xylem formation

The observed delay between leaf and cambial phenology onset (Table 2.2) is generally known for diffuse porous species in temperate regions (Michelot *et al.*, 2012), due to a slow reactivation of cambial activity from the buds to the stem base (Schmitt *et al.*, 2000). Moreover, with a tree height up to 45 m (Table 2.1), *T. superba* is one of the largest trees in the Mayombe and cambial triggering hormones need to migrate from crown to stem base, which can also be a cause of the aforementioned delay. Limited studies on these dynamics are mainly found in temperate regions (Fritts, 1976; Larson, 1994). Precipitation early in the rain season is considered the main driver for *T. superba* growth according to tree-ring analysis (De Ridder *et al.*, 2013). We show in this study that leaf phenology is not directly related to precipitation, so in absence of early rainfall, radial growth through cambial activity can be hampered. In this study, no exceptional drought occurred during the two seasons (Fig. 2.2) and there is a constant lag between leaf and cambial phenology (Table 2.2). However, the exceptional late rain onset for the 2015-2016 season might contribute to the large lag of xylem formation (67 days), compared to previous years (Table 2.2, Supplementary Material Fig. S2.3).

Differences in xylem formation onset between *T. superba* trees were observed by Mariaux (1969) and a distinction is made between valley sites and highlands. It is common that trees of the same species have differing cambial activity in similar environments (Philipson, 1971). Here, the study trees also occur on differing site conditions, albeit close to each other. Interference with other trees causes a shorter growing season (Rathgeber *et al.*, 2011) and the crowding index (Table 2.1) (Michelot *et al.*, 2011) could explain the differences in xylem growing season length.

Missing rings were “live” discovered on two trees in 2013-2014 (Fig. 2.5a) via SDV and confirmed with cambial pinnings. Based on pinning, missing rings were also found in understory trees of the same study area (De Mil *et al.*, 2017).

During the xylem growing season, some growth curves show stagnations (Fig. 2.6a), possibly due to less favourable seasonal weather conditions (Steppe *et al.*, 2015). In contrast to xylem expansion onset and cessation, it is difficult to assign intra-annual fluctuating pattern to water storage or actual rate of xylem growth, because these variations cannot be seen in the results of the pinning experiments, as a monthly resolution is too coarse. The xylem growing curve might still contain a water content component, so the intra-annual pattern of the growth curve (Fig. 6) could also be affected by TWS. However, the magnitude of stagnations in the growth curve is not so pronounced as

generally observed in e.g. Mediterranean regions, where summer and winter are both limiting (Camarero *et al.*, 2010), which can cause intra-annual growth variations. Between the different radii, some circumferential variation in shape of the curve is present, as observed by Robert *et al.* (2014), which results in varying ring widths.

2.4.3 Intra-annual wood traits on a time axis

Linking SDV to actual xylem formation as such is essential to understand seasonal growth. Not only does seasonal growth determine the start and ending of the growing season through cambial phenology (Cuny *et al.*, 2015), it also relates physiology and the xylem with its anatomical structure.

From Fig. 2.7a,b, it is clear that the intra-annual sampling of individual rings, does represent growth increments, but not necessarily equivalent time increments, as stated by Poussart *et al.* (2004). The growth curve is used to convert xylem traits to the time domain (Wimmer *et al.*, 2002; Bouriaud *et al.*, 2005; Skomarkova *et al.*, 2006; Stangler *et al.*, 2016). In order to have an exact timing of when the $\delta^{13}C$ (Soudant *et al.*, 2016), $\delta^{18}O$ and wood anatomical variables occur within a tree ring, this approach is used to fix traits of *T. superba* rings on a time axis, and shows that between-tree cambial phenology differences causes shifts. These shifts would be left unnoticed when averaging between radii or trees per intra-annual position (Fig. 2.7a).

Large variability of the $\delta^{13}C$ profile between trees is also observed in Michelot *et al.* (2011), Skomarkova *et al.* (2006) and Vaganov *et al.* (2009) for temperate species. The within-variability (Fig. 2.7b) of the tree remains largely within limits of 0.5 to 1 ‰ stated by Laevitt (2010) for $\delta^{13}C$. The intra-annual $\delta^{13}C$ variation for *T. superba* in this study is higher than reported for *T. superba* in Cameroon (Fichtler *et al.*, 2010), probably due to the more pronounced dry season in the Mayombe.

Fig. 2.7b illustrates the general trend of the intra-annual $\delta^{13}C$ profile for all trees, and is similar for profiles of temperate (Helle & Schleser, 2004) as well as other tropical trees (Poussart *et al.*, 2004; Verheyden *et al.*, 2004; Fichtler *et al.*, 2010). The increase in $\delta^{13}C$ at the onset of the xylem-growing season is due to the use of starch reserves of the previous growing season, which is typically enriched in ^{13}C (Michelot *et al.*, 2011). The decrease towards the end of the growing season is due to the deployment of current year carbon assimilates to synthesize cellulose (Helle & Schleser, 2004). Fichtler *et al.* (2010) described a similar intra-annual pattern for *T. superba* trees in Cameroon. However, rather

than using a seasonal time, many of these studies assess the isotope pattern in function of ring position, which makes it difficult to link the isotopic response to specific weather events for interpretation, thus missing the more complete environmental effect that can be seen in the $\delta^{13}C$ pattern.

High-frequency variations are observed in the intra-annual $\delta^{18}O$ profile (Fig. 2.7c), without a clear seasonal trend. Variation between radii is higher than the 0.5 – 2 ‰ described in Laevitt (2010). Mixing of various water sources (Gessler *et al.*, 2014), a constant high relative humidity (Roden & Siegwolf, 2012), the close proximity of the Atlantic Ocean and the presence of occult precipitation during the dry season in the Mayombe complicate interpretation. Contrary to the observations of other authors (Barbour *et al.*, 2002, Verheyden *et al.*, 2004a), no positive relation between $\delta^{13}C$ and $\delta^{18}O$ was observed (Supplementary data Fig. S2.6). Although the combined output of $\delta^{13}C$ and $\delta^{18}O$ can contribute to a better understanding of physiological processes (Scheidegger *et al.*, 2000; Colombaroli *et al.*, 2016; Munjonji *et al.*, 2016), the complex forest environment in this study currently restricts the dual isotope model (Roden & Siegwolf, 2012).

Recent techniques that allow to sample at high resolution and select different wood anatomical tissues (Schollaen *et al.*, 2014) could further elucidate the variation in isotopes presented here (e.g. parenchyma sampling vs. fibre sampling). Despite the time-structure conversion of the cellulose stable isotope signals, it is still a complex matter to separate the signal from current year photosynthesis and remobilisation of previous year storage components (Schleser *et al.*, 1999).

As vessel size increases with increasing rainfall (Carlquist, 1966; Michelot *et al.*, 2012) and given their hydraulic function, it is important to reveal their plastic response (Fonti *et al.*, 2010), and therefore, vessels plotted on a time scale in this study. In tropical regions, methods such as tracheidograms (Vaganov, 1990) for conifers or tracheograms for angiosperms (Schume *et al.*, 2004; Abrantes *et al.*, 2013) are less accurate due to the more subtle seasonality presented here, causing non-synchronous cambial activity of individual trees, which is also observed in temperate regions (e.g., Bouriaud *et al.*, 2005; Rossi *et al.*, 2008; Hetzer *et al.*, 2014), but to a lesser extent. In this study on some rings two peaks are seen in the vessel pattern around February. This could be partially explained by a decrease in xylem growth rate (Fig. 2.6) which might induce a decrease in vessel size and frequency: within the rainy season, a small decrease in precipitation is observed around February (Fig. 2.2a), which is common for the Mayombe (Donis, 1948; Mariaux, 1970; Lubini, 1997; Couralet *et al.*, 2013).

Parenchyma area (Fig. 2.9) is related to vessel area because the axial parenchyma is centred around the vessels. The main function of parenchyma is storage (Beeckman, 2016), and while parenchyma is being explored as a climate sensitive trait (Olano *et al.*, 2013), a mechanistic understanding of its usage as a proxy is still lacking.

Although they do not have a general hydraulic function such as in conifers, fibres are increasingly being considered because they account for a significant part of the wood volume and are more continuously present along the ring width (De Micco *et al.*, 2016), rather than vessels or parenchyma. Towards the end of the ring, the fibre lumina get smaller and form the tree ring boundary, which is the key descriptor of the tree ring boundary in *T. superba* (De Ridder *et al.*, 2013), marking the end of the season. The aforementioned shifts can have important implications for tree ring analysis. The study design presented in this chapter aimed at monitoring 4 trees from the same species and from different sites/ages, and sheds new light on the variability in phenology and traits, which needs to be taken into account in the design of future studies.

2.5 Conclusion

Assessing the plasticity of xylem traits from pith to bark and linking them to the past is less evident in tropical trees. In this study, we combine radius dendrometer measurements, cambial pinning data, cellulose stable isotope and wood anatomical traits on mature forest trees in order to fix xylem traits on a time axis via phenology. A concept is proposed linking real time measured variables on a time axis, and wood traits on a distance axis.

Variation in leaf phenology timing is observed between trees, but this is consistent during the monitored growing seasons, and all trees have been shown to flush before the rain onset. Leaf abscission only starts late in the dry season, showing the brevi-deciduous character of *T. superba*.

Leaves start developing irrespective of precipitation, which has important consequences concerning tree vulnerability to climate change: when trees flush, transpiration starts, irrespective of the weather conditions, and in further absence of rainfall, could lead to the loss of turgor at the cambium, followed by decreased growth and eventually tree decline.

Wood formation starts well in the rainy season, and when looking at the cambial dynamics throughout the season, our data show that some intra-annual variation is present in the xylem growing curves which affects the interpretation to wood anatomy and $\delta^{13}C$ or $\delta^{18}O$

isotopes: differing xylem growing periods occur due to varying leaf onsets which cause a different environmental response that will be imprinted differently in the wood structure, causing intra-annual traits not to overlap. The nonlinear weighing of xylem formation (Schollaen *et al.*, 2014), and thus, cambial phenology, should be considered as well, as shown in this study.

Converting wood traits on the seasonal time domain is essential for proper interpretation of the isotope signal, as well as the wood anatomical variables, since different trees form xylem at a different time.

Intra-annual wood cellulose $\delta^{13}C$ signals follow a pattern that is reported in literature, but can only be assessed correctly on a time axis. $\delta^{13}C$ of tree rings contain information from the previous growing season, possible masking the influence of weather events during the current growth season. Variations in source water as well as further fractionations complicate an interpretation of the $\delta^{18}O$ signal.

Converting intra-annual wood traits to the time domain via cambial phenology, causes multiple trees of one species to be synchronized at the intra-annual level, and could thus enable the estimation of cambial and leaf phenology in the past, based on wood anatomy.

We further encourage *in situ* manipulative experiments (Cavaleri *et al.*, 2015; Zuidema *et al.*, 2013), and the effect on the general tree ring structure in the tropics since these could improve tropical tree-ring analysis, and perhaps, further disentangle tree response based on intra-annual wood traits. This would allow to assess more continuous, seasonally resolved time series of wood traits from pith to bark, rather than discretizing to the annual level and considering only ring width in tree ring analysis.


LIMBA
Terminalia superba
CPA
BOVEN
DESSUS
NORD
TINEA
MRAC
MAR.
TINERA
JUIN
JUILLET
AUG
SEP
NOV
DEC
JAN
FEV
MAI
OCT
AVRIL

3

The contribution of vessels parenchyma and fibres in the intra-annual density profile of *T. superba*.

Tom De Mil, Yegor Tarelkin, Joris Van Acker, Hans Beeckman, Jan Van den Bulcke

Preface Chapter 3

In Chapter 2 isotopical and anatomical variation within the tree ring boundaries of T. superba, was observed and it was concluded that cambial and leaf phenology are key in assessing this variation. However, vessel and parenchyma measurements are difficult to up-scale as they are often tedious.

While visually assessing the T. superba stem disks of Chapter 2 (Fig. 3.1), and given the observation of intra-annual wood traits in Mariaux (1967) and De Ridder (2013), intra-annual density fluctuations (IADF's) could be seen in several rings. These IADF's are somehow similar to the fluctuations observed in conifers from temperate and Meditteranean regions, but with a more complex variation of vessels, parenchyma and fibre fluctuations.

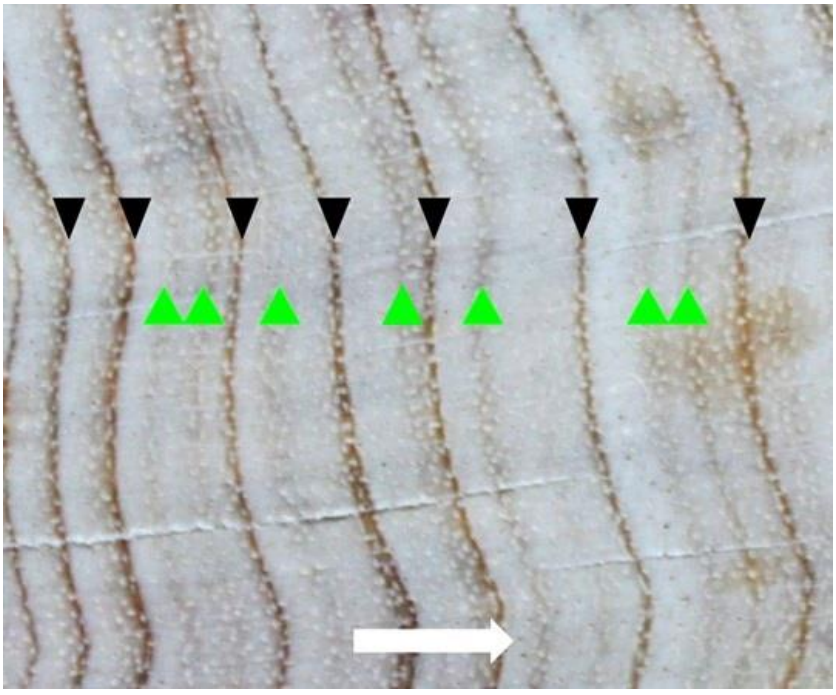

Figure 3.1 Image of a T. superba stem disk surface, showing true ring boundaries (black triangles) and intra-annual fluctuations (green triangles). The white arrow is the growth direction.

Variations in the density profile of T. superba have been discussed in De Ridder et al. (2011), but whether these intra-annual variations of the density profile are due to vessel frequency (Chapter 2), or fibre lumina/wall changes was not discussed and is therefore in the topic of the following chapter.

3.1 Introduction

Wood specific gravity or wood density is an integrating variable (Chave *et al.*, 2009) and a property defined by chemical and anatomical traits (Lachenbruch & McCulloh, 2014). In general, wood density is assessed in the framework of tropical ecology and carbon dynamics (Chave *et al.*, 2009) within and across species. Moreover, the evolution of wood density from pith to bark can vary considerably (Bastin *et al.*, 2015) and most variation can occur between ring boundaries due to seasonal dynamics of wood formation (Bouriaud *et al.*, 2005).

The physiologically relevant proxy Maximum Latewood Density (MXD) is, for instance, used to reconstruct summer temperature (Briffa *et al.*, 1998), and other density-based parameters are explored as well (Bjorklund *et al.*, 2014), to improve climate reconstructions, in addition to the use of tree ring width. Intra-annual wood density profiles can also be related to cambial dynamics (Bouriaud *et al.*, 2005; Vaganov & Shashkin, 2006).

To obtain such detailed wood density values, X-ray densitometry is a well-established tool (Polge, 1966). The tool was shortly after its development used on Aucoumea klaineana (Mariaux, 1967) and was further elaborated on *Terminalia ivorensis* (Nepveu, 1976), in order to delineate ring boundaries or to assess wood quality. Recently, newer techniques were developed relying on 3D rather than 2D data. X-ray CT microdensitometry is a technique in order to obtain large datasets of density profiles (Bergsten *et al.*, 2001; De Mil *et al.*, 2016). X-ray CT microdensitometry is performed in 3D, and analysis ranges from submicron level (Van den Bulcke *et al.*, 2009) to coarser resolutions to perform tree-ring analysis (Van den Bulcke *et al.*, 2014; Steffenrem *et al.*, 2014).

Wood density does not have a direct link with the environment, but the underlying wood anatomical traits do (Lachenbruch & McCulloh, 2014). At the intra-annual scale, the link between density and wood anatomy is straightforward for coniferous species: size of lumina, width of cell wall of tracheids determine the density variation in a ring. The underlying anatomical signal is, however, more complex for angiosperms due to the combination of vessels, parenchyma and fibres that vary within the tree ring to a certain extent. Anatomical decomposition of angiosperm wood has been reported across species (Zieminska *et al.*, 2013), yet unravelling a density profile in its components has rarely been done. Attempts to disentangle intra-annual profiles into vessels, parenchyma and fibres have been reported for *Quercus petraea* (Guilley *et al.*, 2002) and in the tropics for *Gmelina*

arborea (Moya Roque & Tomazello-Filho, 2007) but are understudied for many tropical species.

Specifically for the tropics, the anatomical decomposition of a microdensitometry profile in different proportions of vessels, parenchyma and fibres would increase our knowledge on seasonal tree response. Therefore, it is important to see how variation in the intra-annual density profile is explained by anatomy and in order to use density as a proxy for intra-annual wood traits. In this exploratory chapter, we aim at bridging the more tedious quantitative wood anatomy of Chapter 2, with the more high-throughput and uniform method of X-ray CT microdensitometry. We hypothesize that density profiles are suitable for tropical angiosperm trees, and that they can be related to intra-annual wood traits.

We use *T. superba* as a reference species (De Ridder *et al.*, 2011) (Fig. 3.2). Vessels, parenchyma and fibres were measured manually on microsections and compared to X-ray CT scanning at the resolution of 35 µm and a resolution of 110 µm. The latter "low resolution" approach allows to decrease processing time and increase the amount of samples that can be processed.

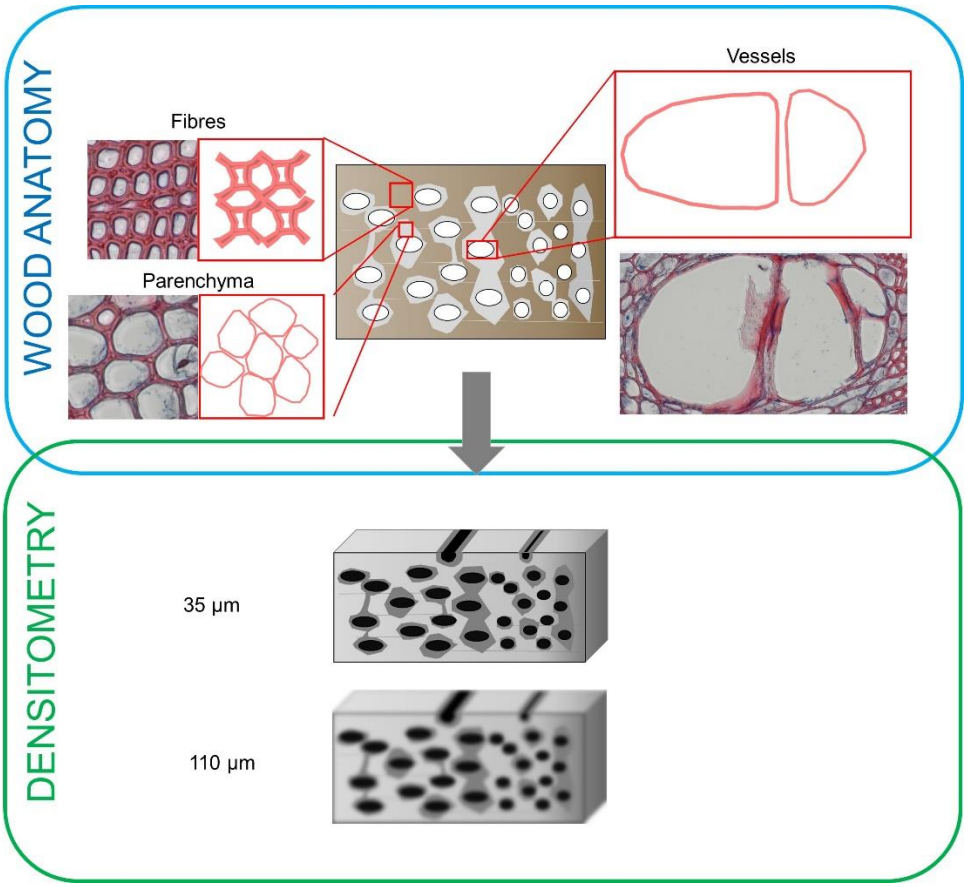

Figure 3.2 Framework of the study: main wood anatomical components are measured on microsections (cell wall stained with safranin, red colour) and compared to the X-ray CT microdensitometry profile at 35 µm and 110 µm.

3.2 Materials and Methods

3.2.1 Wood anatomical measurements

The two live-monitored rings of *Terminalia superba* (T2 & T4) individuals from Chapter 2 were used. Other microsections were of insufficient quality for fibre wall measurements, or the rings were too small to measure a sufficient number of vessels.

Microsections were stained with safranin and alcian blue, and imaged (StreamMotion, Olympus, Japan) on a scanning stage (SCAN 100 x 100, Märzhäuser Wetzlar, Germany) with a camera (UC30, Olympus) mounted on a microscope (BX60, Olympus, magnification 5X). Vessel frequency and vessel/parenchyma area were measured on x5 magnification images in ImageJ (Schneider *et al.*, 2012) via manual selection and the magic wand tool. Axial parenchyma was delineated manually via polygon drawing (cf. Chapter 2).

Fibre lumina and wall thickness were measured on images with x20 magnification via a method based on pattern recognition, as part of the PhD work of Yegor Tarelkin. Briefly, individual cells were automatically identified based on criteria such as the size, the shape and the surrounding pixel intensity variation. We followed the protocol proposed by Kennel *et al.* (2010): filtering, segmentation, tracking and measurement. Image filtering aimed to prepare the image for analyses by reducing the noise. With segmentation individual cells were detected and labelled (Fiorio & Gustedt, 1996; Wu *et al.*, 2005). Tracking allowed connecting different cell's centroids to form the longest chains in the specified direction by using a nearest neighbours (KNN) algorithm, applied on the coordinates of each centroid in the image. The distance used in the KNN algorithm was a combination of the Euclidian distance and the angle related to the horizontal direction. Pixel intensity was then analysed along the path created by the tracking, followed by measuring the width of increase and decrease of the signal. Pixels with high values corresponded to fibre lumina and low values to cell walls. Implementation was based on the Scikit-image (van der Walt *et al.*, 2014) and Scikit learn (Pedregosa *et al.*, 2012) libraries.

3.2.2 X-ray CT densitometry

Increment cores, with a diameter of 6 mm, were sampled on the same position of the microsections. The cores were 3D scanned at 35 µm (helix tomography, see Van den Bulcke *et al.*, 2014) and at 110 µm (cone beam tomography) using the NanoWood CT facility (Dierick *et al.*, 2014), developed in collaboration with XRE (www.XRE.be). The scanned images were reconstructed (GPU GeForce GPX 770 4GB) with the Octopus reconstruction software package (Dierick *et al.*, 2004; Vlassenbroeck *et al.*, 2007; licensed by InsideMatters: www.insidematters.eu).

3.2.3 Statistical analysis

In order to decompose the intra-annual density profile, the fraction of each wood anatomical component at every position should be known. To obtain this, the measurements of the anatomical variables were rescaled at the X-ray CT resolution of 35 µm. Variables used in analysis are (i) wood density, (ii) parenchyma area, (iii) vessel area, (iv) fibre wall fraction and (v) position within ring. Subsequently, pairwise Spearman's rank correlation coefficients were calculated among the variables.

3.3 Results

3.3.1 Density profiles

All rings of *T. superba* have a gradually increasing density trend of the ground tissue frequently interrupted by parenchyma banding and vessels (Fig. 3.3) that lower the wood density. On the X-ray image, the tree ring boundary can be delineated.

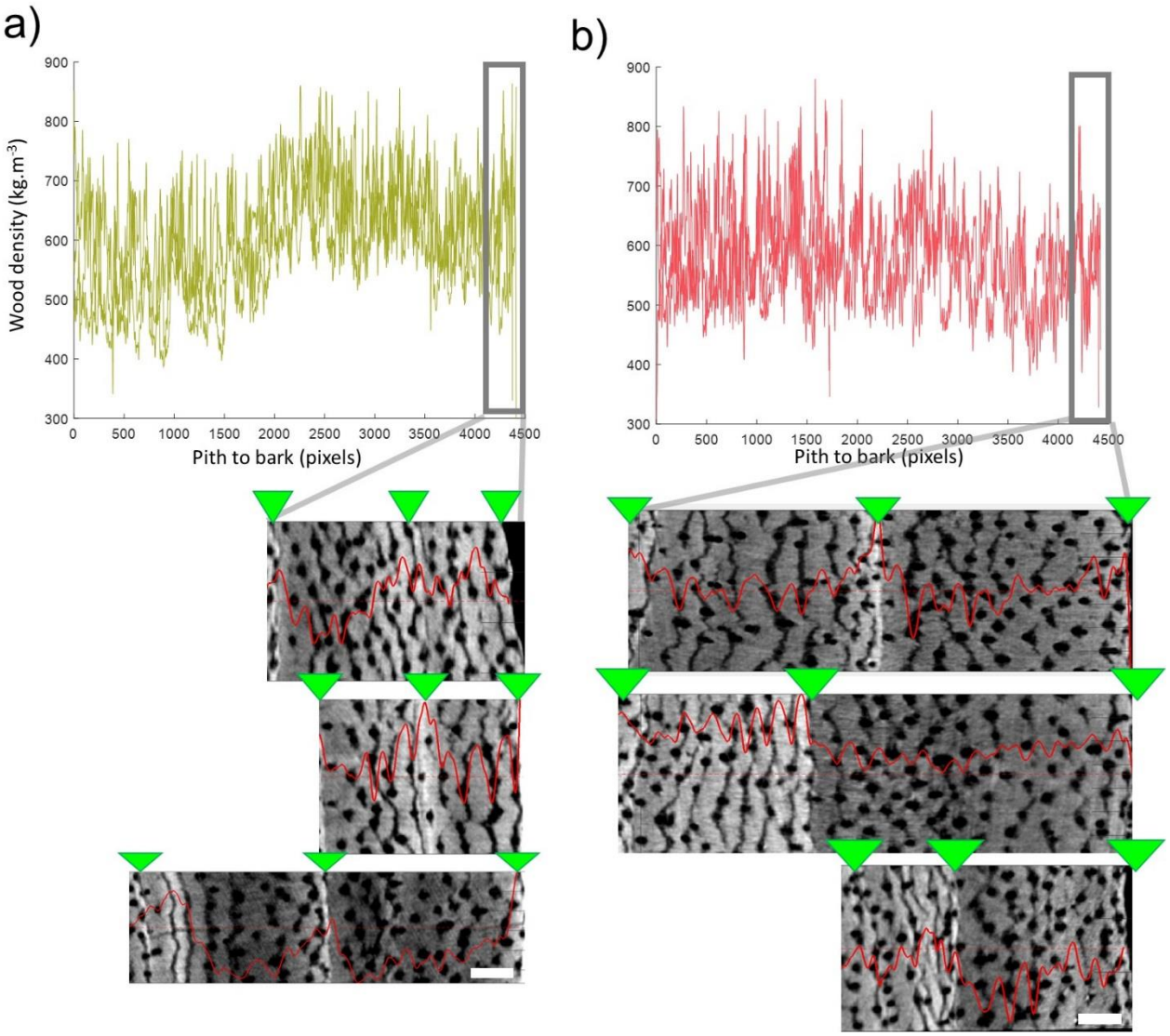

Figure 3.3 X-ray CT scans of increment cores, where the intra-annual profiles of the last two rings of a) T2 b) T4 T. superba trees were compared to its wood anatomical components. Tree ring boundaries marked with green rectangle.

Average parenchyma area was 12.57 %, vessel area was 6.19 % and the density varied between 500 and 750 kg/m³.

3.3.2 Relation wood density and wood anatomical traits

Vessels and parenchyma have an inverse relationship with density and are mainly related to each other. Fibre wall variation is positively related to wood density, whereas position is correlated with the fibre wall fraction (Table 3.1).

*Table 3.1 Spearman correlation coefficient matrix, * P<0.05, ** P<0.01.*

	Wood density	Parenchyma area	Vess. area	Fibre wall fraction	Position
Wood density		-0.1297*	-0.2531**	0.2645**	0.0807
Parenchyma area			0.4217**	-0.0940	0.0797
Vessel area				0.0031	0.0215
Fibre wall fraction					0.1571*
Position					

There is a clear trend with inverse relation between vessels / parenchyma and the wood density profile.

From Fig. 3.4, the profiles scanned at 110 µm still provide a sufficient resolution to see the variation of intra-annual wood traits, as well as tree ring boundary detection.

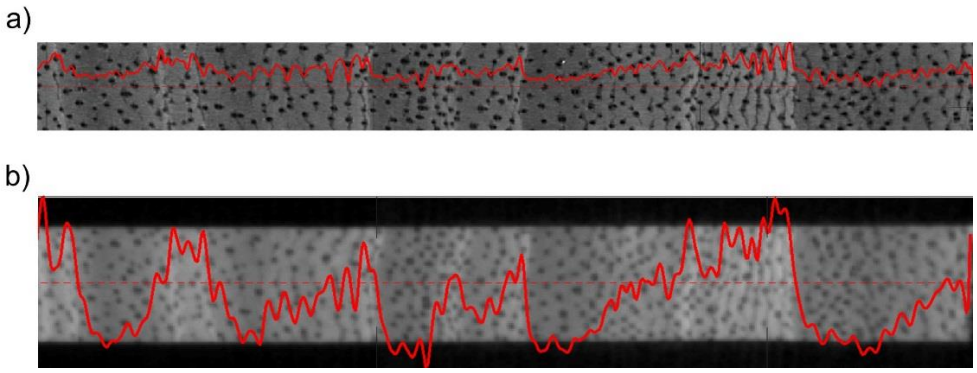

Figure 3.4 X-ray CT images of a T. superba core,scanned at (a) 35 µm resolution, (b) 110 µm. Density profile is superimposed.

3.4 Discussion

A positive relation between fibre fraction and density was shown for *T. superba* in Ghana by Kessels-Dadzie *et al.* (2016). The association between vessels and parenchyma (Table 3.1) is due to the aliform or confluent nature of the axial parenchyma surrounding the vessels (De Ridder, 2013). In accordance with Zieminska *et al.* (2013) and Moja Roque & Tomazello Filho (2007), fibre wall fraction is positively related to wood density in this study. Axial parenchyma is negatively correlated with wood density, which concurs with Martinez-Cabrera *et al.* (2009).

Several other species will be needing the same exercise to understand the anatomy underlying their density profile, and this will also determine whether or not that species is

useful for density profiling at that resolution. The tree-ring boundary is generally known to be a density fluctuation due to the fibres (De Ridder *et al.*, 2011), thus allowing to perform tropical tree-ring analysis with densitometry (Brienen *et al.*, 2016). The positive relation between position and fibre wall fraction in this study (Table 3.1.), further confirms the variation of fibres wall/lumina as the ring is formed throughout the season. IADF's due to drought will also likely be reflected in the density profile, as vessel size could decrease and fibre wall fractions could increase. Moreover, although not shown in this analysis, in addition to fibre wall fraction, fibre proportion is merely the opposite of the sum of vessel and parenchyma fraction. Thus, not only the internal fibre variation, but also the fibre proportion will determine wood density at a certain position, and is inverse to the vessel and parenchyma pattern**.**

The variation in wood anatomy due to banding of parenchyma, combined with the superimposed effect of the fibre variation is observed in this study for *T. superba* and has been described in De Fay (1992) for *T. ivorensis* and is clearly seen on the density profile and X-ray CT images of *T. superba* in Fig. 3.4. Parenchyma and vessels cause highly oscillating curves hampering tree-ring analysis (Mariaux 1967 in Worbes 2002), which could also be deduced from our results based on Spearman's rank coefficients and is generally seen on the entire density profile (Fig S.1). Moreover, next to the monitored years, parenchyma and vessels fluctuate strongly from year to year, and in some years they even mask the typical trend of density increase towards the tree ring boundary when only investigating the 1D density profile. It should be noted however that on 2D slices through the 3D CT volume (eg. Fig. 3.3) , tree ring boundaries still can be delineated visually. Therefore such images still allow tree ring boundary demarcation.

Delineating ring boundaries should, however, not be the only criterion to assess wood density profiles. Vessels and parenchyma are strongly related, their fluctuations could reveal relevant information concerning physiology as well.

Although the features are more mixed, the profile scanned at 110 µm still provides sufficient information on intra-annual traits, thus can be further used as a screening resolution.

3.5 Conclusion

Intra-annual wood traits are increasingly being used to assess tree performance in the past. Densitometry is mainly applied for creating density profiles of conifers in temperate regions, but is rarely performed on tropical angiosperms. Whilst a promising technique, a decomposition of the density profile in its anatomical traits is necessary for a correct interpretation of the intra-annual density fluctuations.

As a proxy for wood anatomical traits such as vessels, parenchyma and fibres, X-ray microdensitometry is proposed as a technique that can be performed in a fast and non-destructive way on intra-annual profiles for rings of *T. superba.*

This study showed that microdensitometry is able to map intra-annual traits and gives an estimation of the intra-annual variability of vessels area, parenchyma area and fibre variation via principal component analysis. For *T. superba*, the fibre wall fraction determines the largest positive variation in the intra-annual profile. Vessel area as well as parenchyma are negatively related to the intra-annual density profile. The general upward trend for the fibres, is interrupted by the parenchyma and vessel banding.

The negative relation with vessels and parenchyma, and the positive one with fibres, suggest that microdensitometry is a good proxy for intra-annual wood traits. Moreover, this study demonstrates that rings can be delineated and intra-annual fluctuations in *T. superba* can be detected with X-ray CT microdensitometry.

Preliminary research conducted shows that X-ray CT scanning of rings at 4 µm also allows to derive vessel area, parenchyma fractions and fibre variation (through intra-variation in greyscale values) and provided the same results as the manual measurements. Moreover, based on results of Van den Bulcke *et al.* (2014), vessel-free density can be performed in an automated way. This shows the wide applicability of X-ray CT (Suppl. Material Fig. S3.1.), so that this tool can be applied to multiple scales, from intra-annual level, to the entire pith-to-bark level.


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4

A field-to-desktop toolchain for X-ray CT densitometry enables tree-ring analysis

Tom De Mil, Astrid Vannoppen, Hans Beeckman, Joris Van Acker, Jan Van den Bulcke

Abstract

Disentangling tree growth requires more than ring width data only. Densitometry is considered a valuable proxy, yet laborious wood sample preparation and lack of dedicated software limit the widespread use of density profiling for tree-ring analysis. We present an X-ray computed tomography-based toolchain of tree increment cores, resulting in profile datasets suitable for visual exploration as well as density-based pattern matching. Two temperate (*Quercus petraea*, *Fagus sylvatica*) and one tropical species (*Terminalia superba*) were used for density profiling using an X-ray computed tomography facility with custom-made sample holders and dedicated processing software. Density-based pattern matching is developed and able to formally detect anomalies in ring series that can be corrected via interactive software. A digital workflow allows to generate structure corrected profiles of large sets of cores in a short time span that provide sufficient intra-annual density information for tree-ring analysis. Furthermore, visual exploration of such datasets is of high value. The dated profiles can be used for high-resolution chronologies and also offer opportunities for fast screening of lesser studied tropical tree species.

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Preface Chapter 4

From Chapter 3, densitometry- derived density profiles seem a suitable property for measuring intra-annual traits in T. superba, as such having a positive relation with fibre wall fraction, and a negative with vessels and parenchyma. The general upward trend in the intra-annual density profile is due to fibre variation, whereas the highly varying intra-annual pattern superimposed is due to vessels and parenchyma variation. When the intra-annual profiles (Chapter 3) of the rings in Chapter 2 are plotted on a time axis, it is clear that shifts occur and earlywood and latewood are not entirely overlapping between trees. Next to ring wedging and missing rings, these varying time windows due to intrinsic conditions could be a main cause for weak correlations with climate. However, intra-annual features can be used to match series (Wils et al., 2011) and based on the above, density profiles are suitable to assess intra-annual fluctuations from pith to bark. In Chapter 4, we develop an X-ray CT densitometry toolchain for density profiling of cores in a fast way. Moreover, given Chapter 2 and Chapter 3, a concept is proposed to enhance classic tree ring analysis, based on intra-annual traits, which is especially relevant in tropical trees. As a benchmark, two species of temperate regions, Fagus sylvatica and Quercus petraea, are used to test the methodology.

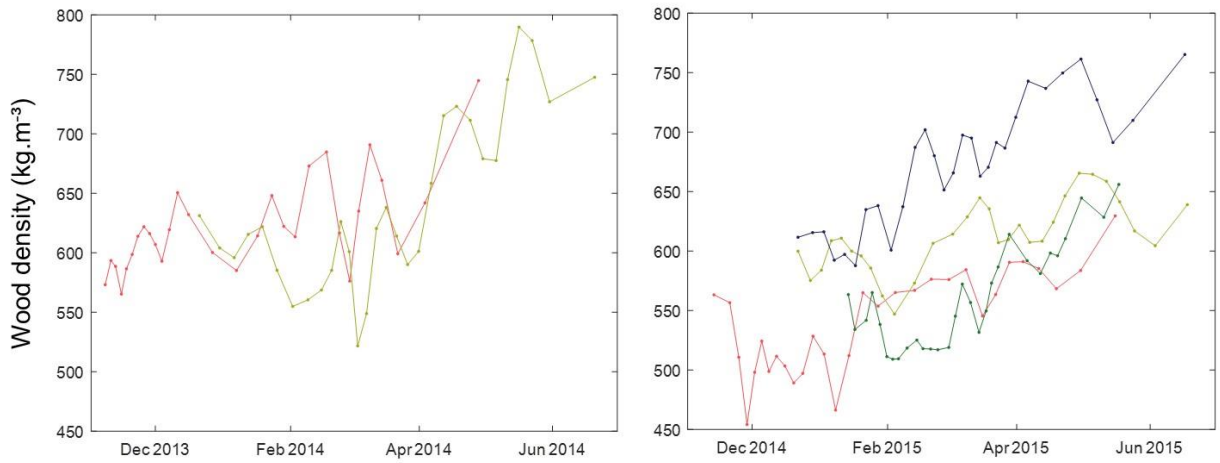

Figure 4.1 The intra-annual density profiles on a time axis for tree T1 (green), T2 (yellow), T3 (blue) and T4 (red) from chapter 2 are shifted in time, which would be masked by pure averaging based on intra-annual distance units.

4.1 Introduction

Quantifying the response of trees to a globally changing environment is essential to predict their future role in the carbon balance (Lewis *et al.*, 2009; Martin & Thomas, 2011). The combined action of intrinsic and environmental factors is responsible for the unique structure of wood, which makes tree rings important natural archives (Vaganov *et al*., 2006). There is an urgent need for more knowledge to increase our understanding on wood formation under changing environments (Worbes, 2002; De Micco *et al*., 2012) which requires methodological improvements (Fonti *et al*., 2010). In addition to ring width, wood density is a promising indicator. It is determined by anatomical and chemical traits (Lachenbruch & McCulloh, 2014) and is a key descriptor of wood (Chave *et al*., 2009). Wood density is defined as mass divided by its volume and can be seen as a proxy for carbon allocation per unit of volume (Müller-Landau, 2004; Plourde *et al*., 2015), as it reflects past climate conditions (Hughes, 2002; Büntgen *et al*., 2010) and is an important property for life history strategies (Preston *et al*., 2006; Swenson & Enquist, 2007; Zanne *et al*., 2010; Poorter *et al*., 2010).

Densitometry is used to quantify density variations that reach the intra-tree ring level (Fritts, 1976). X-ray densitometry by means of radiographies was developed by Polge (1966) and is, together with other systems such as SilviScan (Evans, 1994), widely used as a basic technique for density profiling of tree increment cores. Derived parameters such as Maximum Latewood Density (MXD) have proven to be more sensitive to climate than tree ring width in high northern latitudes (Briffa *et al*., 1998). Features such as intra-annual density fluctuations can be detected with densitometry (Gonzalez-Benecke *et al*., 2015) and are vital for monitoring response to short-term climatic changes (Pilcher *et al*., 1990; Wimmer, 2002; Battipaglia *et al*., 2014), yet can be masked by averaging over the entire tree ring width (Rossi & Deslauriers, 2007). Such density profiles can be used to match within and between individuals, similar to matching ring width series i.e. cross-dating. Density might even be more important than ring width itself in the matching process (Polge, 1966, 1970; Allen *et al*., 2012; Drew *et al*., 2013). Traditionally, cross-dating is the basis of dendrochronology where ring widths are measured on wood cross-sections (core or stem disk), after which patterns of wide and small rings of several trees are visually/statistically matched and anomalies (e.g. missing rings, false rings) removed in order to assign every ring to an exact calendar year (Fritts, 1976).

Next to several existing software packages such as COFECHA (Holmes, 1983; Grissino-Mayer, 2001), the eventual cross-dating is often dependent on personal judgement

(Pilcher *et al*., 1990) and is non-trivial in many regions (e.g. tropical and subtropical latitudes) and often difficult to reproduce (Wils *et al*., 2009) and to formalize. Density profiles thus offer a clear opportunity for improvement in this respect.

Throughout the years, the original idea postulated by Polge (1966), i.e. cross-dating using original density profiles, was only possible on a visual basis and on a limited number of samples. Furthermore, despite the potential high-resolution, the classic technique is costly, time-consuming and still labour-intensive, hampering fast data acquisition and thus the possibility for high-throughput automated tree-ring analysis, although semi-automated density-based delineations of ring width (Mothe *et al*., 1998) and density parameters (Koubaa *et al*., 2002) exist. Furthermore, due to increasing demand for incorporating other sources of information to disentangle tree growth, often involving destructive analyses such as isotope measurements (Schollaen *et al*., 2014) or elemental analysis (Hietz *et al*., 2015) there is a need for digitally archiving structural data before further analysis. There is an ongoing interest in developing new methods for determining wood density in a more detailed and less time-consuming way (Mannes *et al*., 2007) and less dependent on physical manipulations. Recent techniques specifically reducing labour and time costs are high-frequency densitometry (Schinker *et al*., 2003; Wassenberg *et al*., 2014) and medical X-ray Computed Tomography (X-ray CT) (Steffenrem *et al*., 2014). Blue intensity measurements based on optical images are also being developed as a more cost-effective method for MXD in conifers (Rydval *et al*., 2014). Although an integrative approach of high-throughput density profiling combined with the application of software to apply full densitometry information for objective cross-dating is still lacking. In this study, the Nanowood X-ray CT scanner developed at UGCT (Dierick *et al*., 2014) is used for high-throughput 3D scanning of increment cores to obtain density profiles from pith to bark. De Ridder *et al*. (2011) and Van den Bulcke *et al*. (2014) elaborated on this technique for temperate and tropical species to demonstrate the possibility for tree-ring analysis, taking into account ring and grain directions to obtain structure corrected density profiles.

The aim of this chapter was to establish an X-ray CT-based field-to-desktop toolchain for large sets of increment cores, with accompanying software routines that allow:

Screening and archiving of 3D digital wood samples before any further physical treatment, followed by batch processing of calibrated re-interpolated density profiles with minimum labour-input.

Automating the process of tree ring registration and formalizing cross-dating through density-based pattern matching of the profiles archived in (i), in order to detect anomalies and explore datasets via built-in graph functions.

We demonstrate the approach by explaining the toolchain for two long-lived deciduous temperate species: sessile oak (*Quercus petraea*) and common beech (*Fagus sylvatica*), as well as a tropical brevi-deciduous tree species limba (*Terminalia superba*).

4.2 Materials and Methods

4.2.1 Sample selection

Q. petraea, *F. sylvatica* and *T. superba* increment cores (5 mm), drilled at breast height, were used for density profiling and tree-ring analysis. 46 cores from *Q. petraea* and 46 cores from *F. sylvatica* were sampled (two cores per tree) in the winter of 2014 at different forest sites in the South of Belgium, specified in Vannoppen et al. (2017). 46 *T. superba* cores (on average three cores per tree) were sampled at the end of the growing season in 2014 in the Luki Reserve, located in a semi-deciduous forest that is part of the southernmost edge of the Mayombe forest, DR Congo (Democratic Republic of the Congo).

4.2.2 Core treatment

All increment cores were put in paper straws after sampling for ease of storage and labelling and remained unwrapped throughout the entire X-ray CT toolchain. The samples were dried for 24 h at 103 ± 1 °C and mounted in custom made cardboard holders for scanning. Current setup allows to store up to 33 intact cores of variable length (up to 50 cm) into one holder. A datasheet that links the sample label with position in the holder allows for batch extraction, and metadata (site, coordinates, etc.) are included for archiving. The sample holders with cores were scanned at a resolution of 110 µm using the NanoWood CT facility (Dierick *et al*., 2014). A sample holder does not fully fit in the field of view entirely and therefore top and bottom part were scanned separately, reconstructed (GPU GeForce GPX 770 4 GB) with the Octopus reconstruction software package (Dierick *et al*., 2004; Vlassenbroeck *et al*., 2007; licensed by InsideMatters: www.insidematters.eu) and digitally stitched resulting in a single greyscale volume of the entire sample holder. All routines to process the greyscale volume, as elaborated on the

Results section, were written in MATLAB® R2015a and are available as a stand-alone package with a graphical user interface and associated graphs.

4.3 Results

A flow-chart of the toolchain is given in Fig. 4.2. Several steps are executed as batch processes, as such minimizing labour cost and increasing speed of analysis.

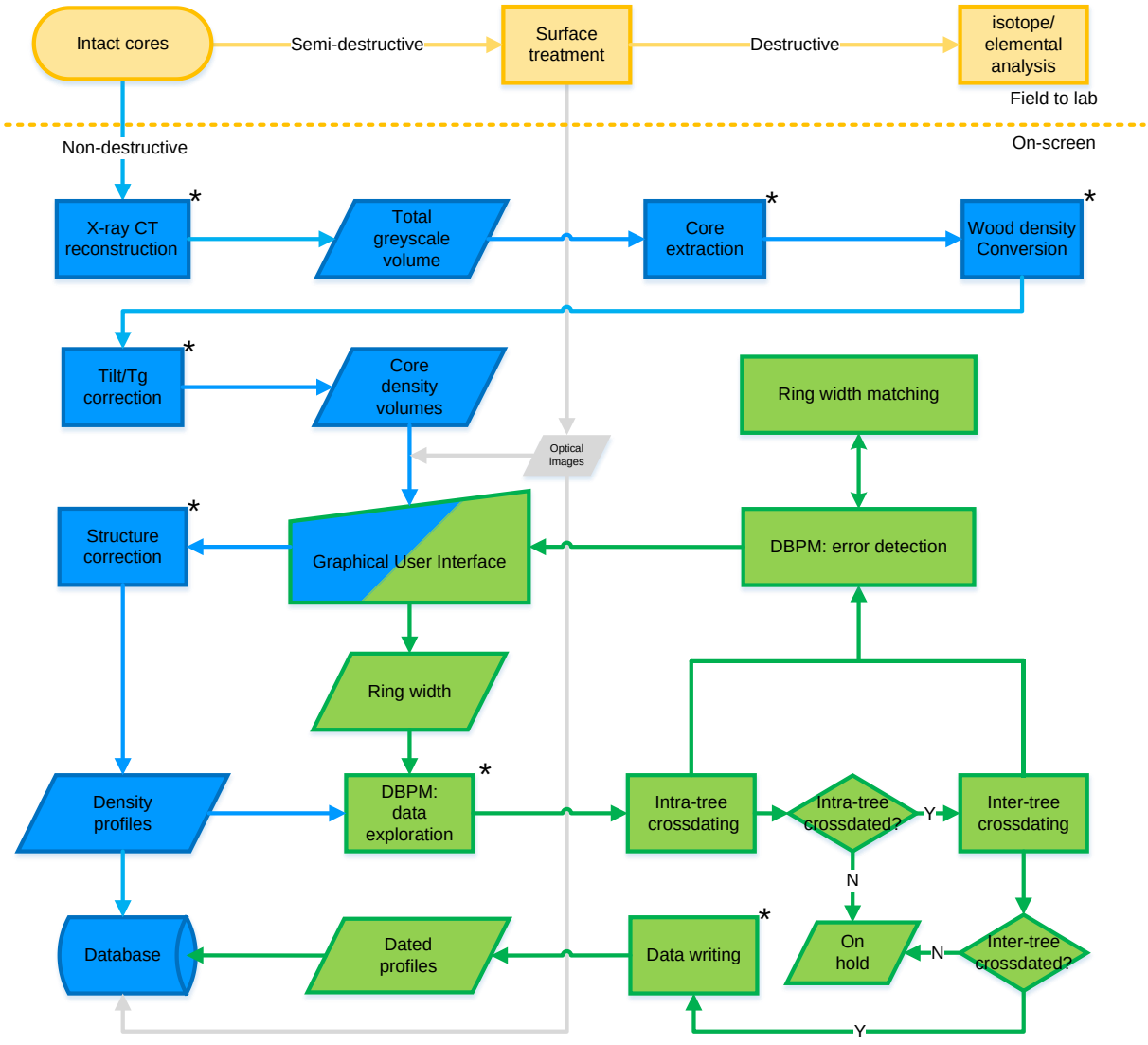

Figure 4.2: Workflow of the X-ray CT toolchain for densitometry and corresponding tree-ring analysis. The density screening/profiling module is presented in blue, the pattern matching module is presented in green. XCT-GUI (hatched) serves as an interface between the two modules where users indicate structure direction and tree rings. Rectangles represent processes, parallelograms data. Processes indicated with (*) refer to batch procedures.

4.3.1 Screening and archiving

50 minutes scanning and 30 minutes reconstruction time per cylinder are required. The reconstructed greyscale volume of the entire sample holder is loaded in the toolbox, all cores are indicated on a cross-section and labelled automatically based on the datasheet (Fig. 4.3a). Each labelled core is then extracted from the total greyscale volume and converted to wood density estimates by rescaling using a reference material with known density (1400 kg m^{-3}) and similar elemental composition as wood (De Ridder *et al*., 2011) and air (1.2 kg m^{-3}) (Fig. 4.3b). Wood density is thus defined as ovendry weight divided by ovendry volume. Each core density volume then needs to be oriented perpendicularly to the grain, which is essential for proper structure direction correction later on. This orientation is automated as a two-step process including tilt and tangential alignment (Fig. 4.3c), avoiding tedious manual rotation of the object in three dimensions. The former aligns the core axis parallel to the X-axis of the global coordinate system. The latter rotates the core in the tangential plane according to the user indicated grain angle based on display previews through the core. Each core density volume is then stored as a single 16-bit multipage TIFF file. These digital cores can then be loaded in the Graphical User Interface (GUI), an extended version of the structure correction module described in Van den Bulcke *et al*. (2014), allowing visualisation of both the transversal (Fig. 4.4) and the radial plane. Cores are first assessed visually in order to exclude cores showing e.g. knots, wound reactions or anomalously suppressed growth.

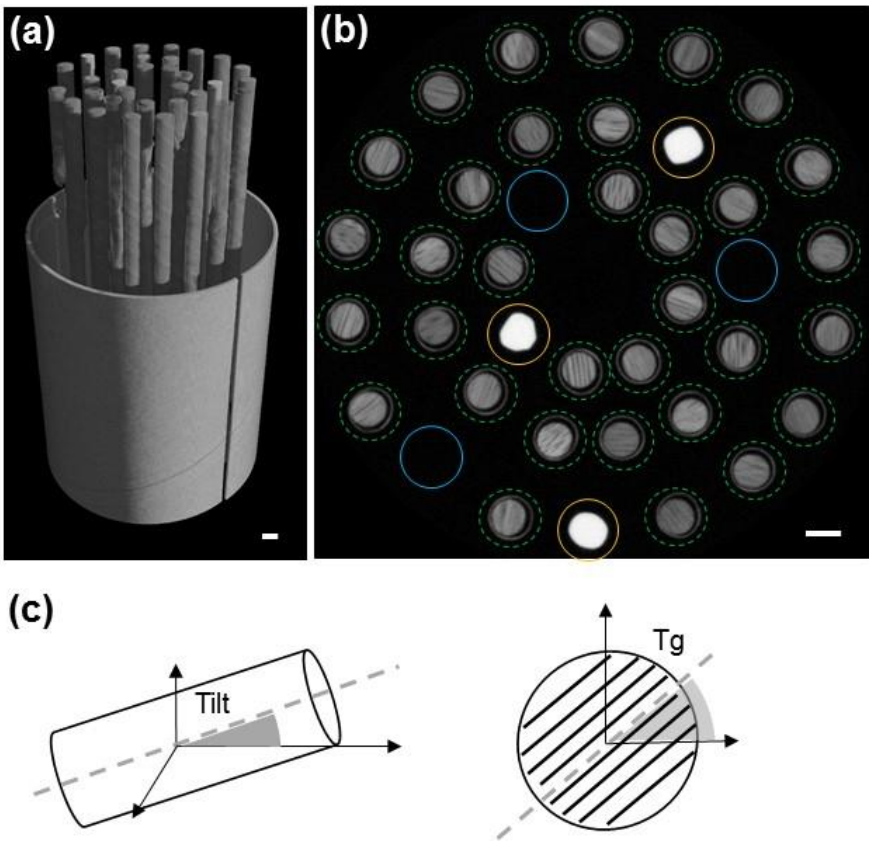

Figure 4.3 (a) 3D rendering of the sample holder volume containing the tree cores (b) Cross section of the volume with indication of the cores (green dotted circles) for batch extraction. Reference material (yellow circles) and air (blue circles) are extracted and used to convert the separate core greyscale values into wood density values (c) tilt and tangential alignment of the core volume for further analyses. Scale bar: 5mm.

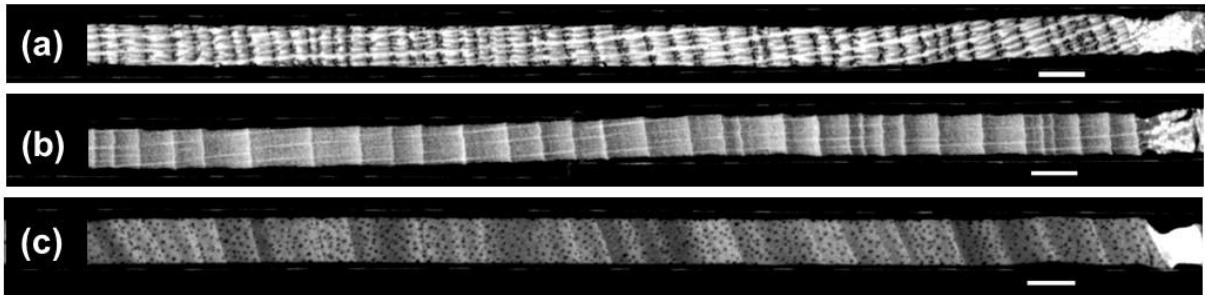

Figure 4.4 Visual screening of the X-ray CT images allows fast inspection of the wood surface: transversal view of a (a) Quercus petraea, (b) Fagus sylvatica and (c) Terminalia superba core. Scale bar: 5mm.

Subsequently structure direction is corrected for, tackling (i) ring curvature and (ii) radial variations in grain direction (Supplementary Information Fig. S4.1). This can be accomplished by indicating the main structure direction. In classic tree-ring analysis, ring

angle is accounted for by rotating the sample during tree ring width measurements, while grain deviations in the radial plane are not considered. For coniferous tree species with a clear ring structure this correction can be performed automatically, for details see Van den Bulcke *et al*. (2014). Other tree species with more subtle structures at the tree-ring border (e.g. tropical tree species) require manual indications. Once the structure direction is indicated, a re-interpolated density profile is calculated excluding the paper straw and surrounding air using a morphological operator (Supplementary Information Fig. S4.2).

This approach will segment the wood volume from the background, such that no manual cropping of the volume is necessary and all the density profiles are automatically extracted. Re-interpolated density profiles from pith to bark and the average density trend, are presented in Fig. 4.5 for the three wood species studied. For *Q. petraea* and *F. sylvatica*, a rather constant density from pith to bark is observed, while for *T. superba* an increasing density occurs.

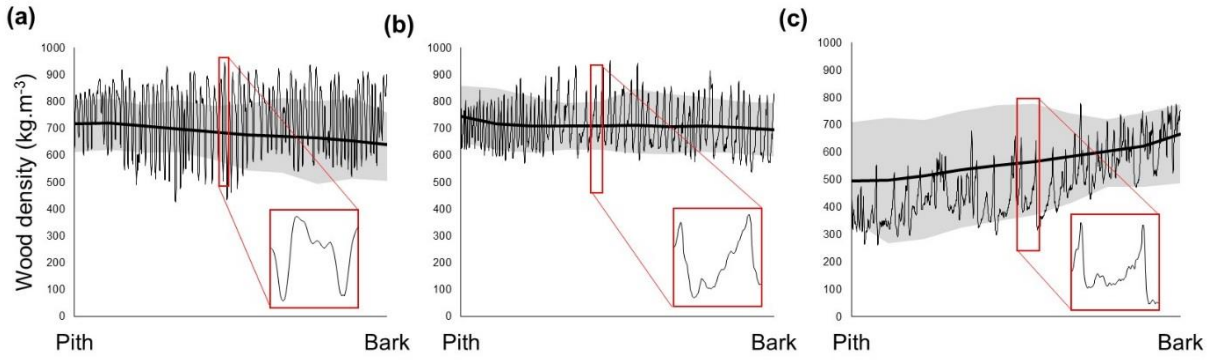

Figure 4.5 Averaged wood density trend (smoothed line, error bands show one standard error) and single density profile from pith-to-bark for (a) Quercus petraea, (b) Fagus sylvatica and (c) Terminalia superba. The latter shows an increase in density from pith to bark. Magnification of a single ring illustrates the tree ring boundary type.

These profiles can then be used to automatically indicate tree rings or fine tune manual indications made in the GUI. This detection is based on a user-specified threshold for minima, maxima or inflection points in the density profile, a decision which should be based on the tree ring boundary type (Worbes, 1989): *Q. petraea* is a ring-porous species with tree ring boundaries defined by minimal density (onset early wood vessels), while *F. sylvatica* and *T. superba* are diffuse-porous characterised by a clear density peak at the ring boundary. The maximum density is caused by a decrease in number of vessels for *F. sylvatica*, and smaller thick walled fibres for *T. superba*. Errors can easily be removed

interactively and if manual indications of the tree ring boundaries already existed, a fine tune function can be used to shift the indications to the extrema or inflections of the density profile.

A smoothing filter can be applied to each density profile in order to reduce high frequency details originating from wood anatomical features such as e.g. abrupt changes between vessels/parenchyma and fibres and crystals. Flatbed images serve as a complementary archive and can easily be coupled to the X-ray images (Supplementary Information Fig. S4.3).

4.3.2 Tree-ring analysis

Density-Based Pattern Matching (DBPM) is illustrated for two *F. sylvatica* core profiles, referred to as R and Q (Fig. 4.6), but is analogous for comparison of multiple cores.

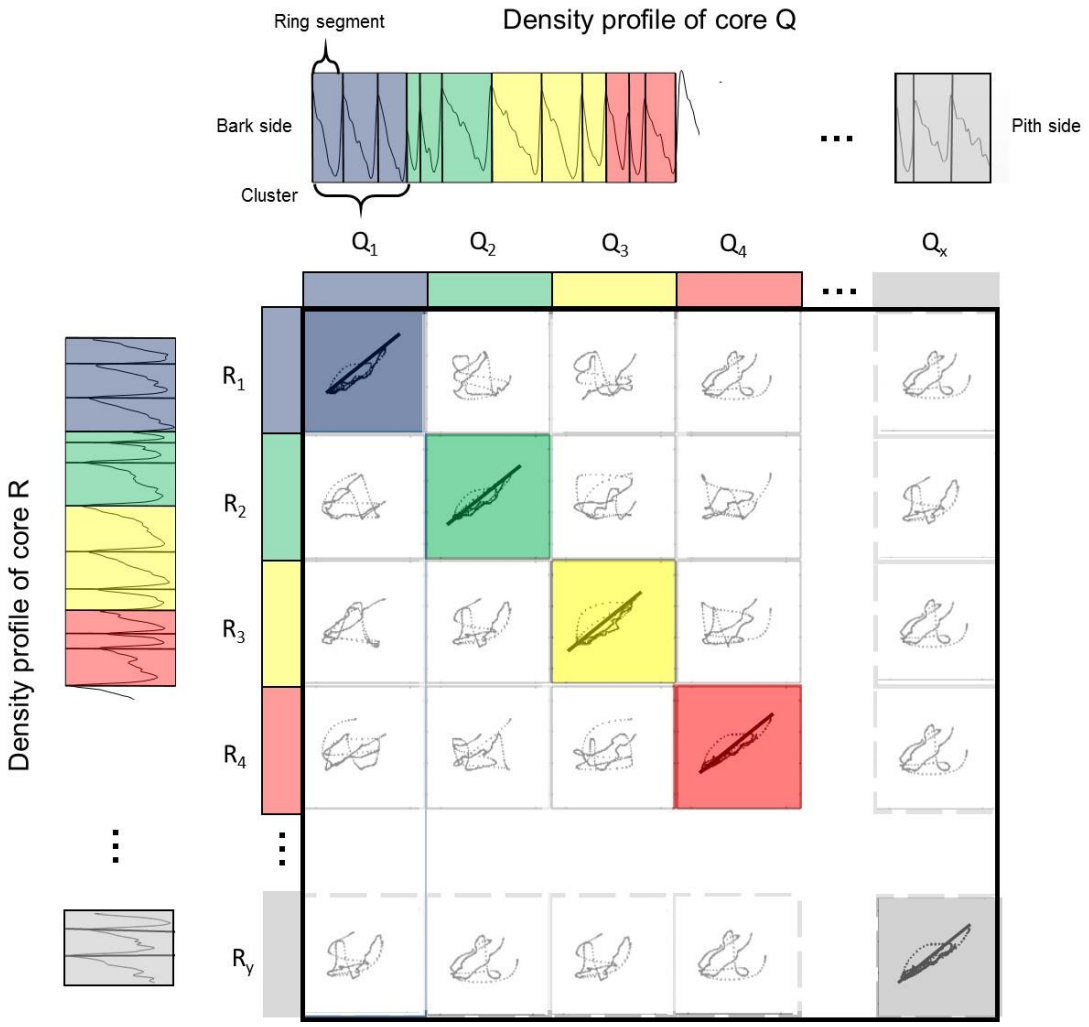

Figure 4.6 Density profiles of Fagus sylvatica core R and Q are segmented based on ring indications. In this example, rings are grouped by 3 to form resized clusters to assess similarity based on a correlation matrix. High correlations are noted on the diagonal, which is the basis of the similarity measure.

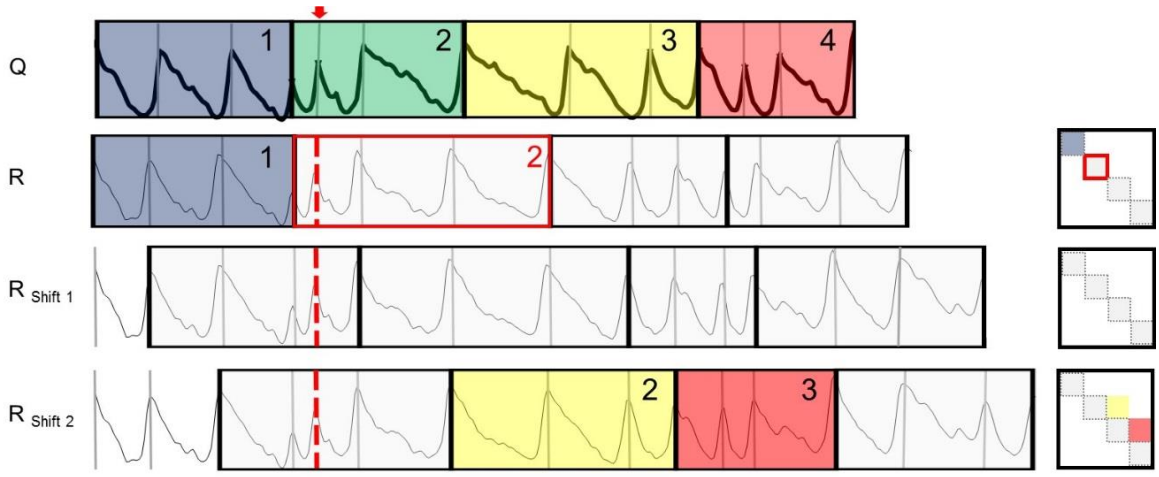

Figure 4.7 Illustration of the shift procedure of the Density-Based Pattern Matching (DBPM) for core R and Q with one missing indication (red dotted line) in cluster two of core R (cluster size: 3). Clusters are shifted with one ring (R_{shift1}) and two rings (R_{shift2}) and ring segments are merged into new clusters and correlation is recalculated. Coloured clusters represent matched zones between the cores.

For each core, tree ring boundaries, manually or automatically indicated in the GUI, are used to divide the density profile into separate ring density profiles. Subsequently, these consecutive ring profiles are merged into clusters with a user-specified size. Clustering thus allows to include sufficient density information in order to reduce the number of false positives while matching. All cluster sequences are correlated using Pearson correlation. This requires to resize the clusters to equal length and standardise density, emphasising relative differences in wood density, rather than absolute differences. DBPM starts arbitrarily at bark side. If tree rings are indicated properly, i.e. without any false or missing rings and cross-dated, high correlations will appear on the diagonal of the R-Q correlation matrix, which is a similarity measure reflecting the mutual structural synchronicity along the growth axis. To account for false/missing rings or errors in ring indications (further referred to as anomalies), a method is proposed where ring segments are shifted and re-clustered, followed by a recalculation of the correlation matrix. Fig. 4.7 illustrates the procedure for the same two cores as in Fig. 4.6, with a missing indication in the second cluster of core R. The initial situation, without any shifts, will only cause matching of the first cluster of Q and R. All further clusters from Q and R cannot be matched due to the anomaly. The maximum shift is cluster size-1 because the initial situation is re-attained when the number of shifts equals the cluster size. After shifting one ring (R_{shift1}), no clusters are correlated anymore with Q because the positions are moved. Shifting two ring segments (R_{shift2}) results in a match for the third and the fourth cluster of core Q with the second and third cluster of core R. Evaluating all shifts results in localisation of the cluster that contains the error. The approach thus allows to locate all ring indications based on

density and leads to actual matching (cross-dating) by tracking the anomalies and removing them using the GUI. False rings or errors in ring indications can be interactively removed. Missing rings are marked and labelled in the GUI and will be accounted for in further analysis. Broken cores, cracks, scars and other artefacts can be labelled in order to exclude these for further processing (Supplementary Information Fig. S4.4). Different sampling dates are accounted for during the DBPM procedure.

The software allows DBPM parameters and the associated plots to be verified with classic ring width curves and tree ring descriptive statistics such as the corrected Gleichläufigkeit criterion (Buras & Wilmking 2015) (Supplementary Information Fig. S4.5).

Simultaneously, core volumes are loaded in the GUI to remove/add rings, depending on the abovementioned assessment procedure, after which all statistics are updated. Once all cores are cross-dated, a batch operation exports the density profiles, associated ring width series and derived parameters such as mean density per ring or MXD for use in other statistical or tree-ring analysis software packages. During the procedure, the user can visually assess multiple profiles simultaneously.

DBPM was applied on semi-automated ring indications of undated profiles which resulted in effective cross-dating of all density profiles of *F. sylvatica* and *Q. petraea*, and was verified with ring width statistics. A matrix output for the two species illustrates the difference in number of cluster matches before (Fig. 4.8 a,c) and after (Fig. 4.8 b,d) cross-dating with cluster size 3. On specific radial positions, a higher number of cluster matches is noted for cross-dated cores of *F. sylvatica* and *Q. petraea*. For *T. superba*, only medium-sized rings provided sufficient density information to be matched between cores. Due to a strong age trend, very small rings occur in the outermost core section, which complicated exact dating of the *T. superba* profiles (Fig. 4.8 e,f).

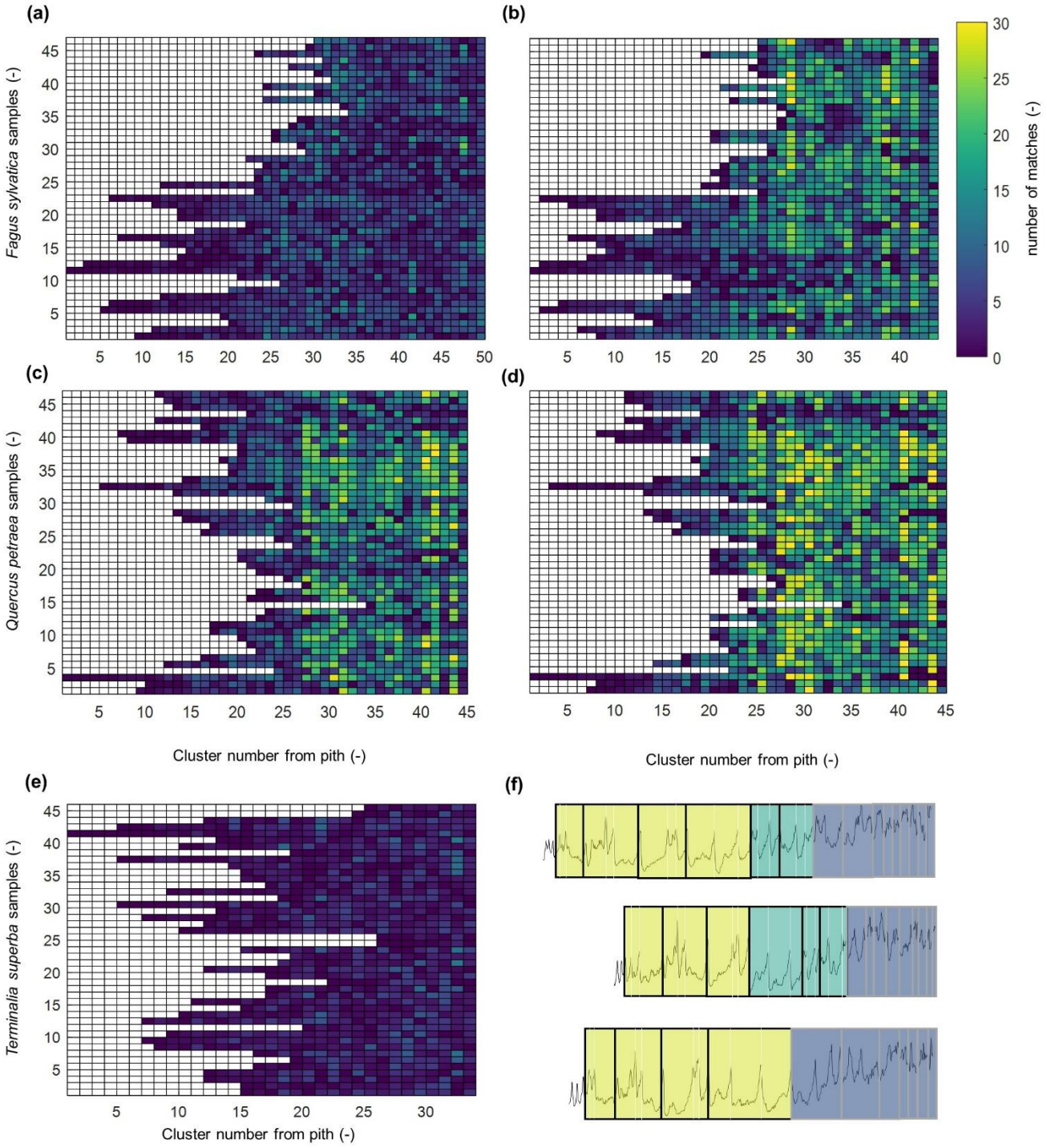

Fig. 4.8 Matrix plots of the number of density-based matched clusters (a) before, and (b) after cross-dating of Fagus sylvatica in function of the sample and the position of the cluster within the sample. (c) and (d) identical for Quercus petraea. The colour scale of each element represents the number of clusters that it has in common with other profiles in that same cluster number. Same colour scale is used for the three species. After cross-dating, a higher numbers of clusters is found (cluster size: three). Number of clusters is different before and after cross-dating due to adding/deleting ring indications. (e) For Terminalia superba few matches are found due to (f) difficult matching of small rings.

Subsequently, to interpret the DBPM, the matched clusters from Fig. 4.8 should be assigned to calendar years: the number of density matches per year is plotted for all the samples, allowing to assess possible common intra-annual density signals (Fig. 4.9 a,b). Additionally, matrix plots of ring width and density parameters of cross-dated cores can serve as data exploration to look for signal patterns.

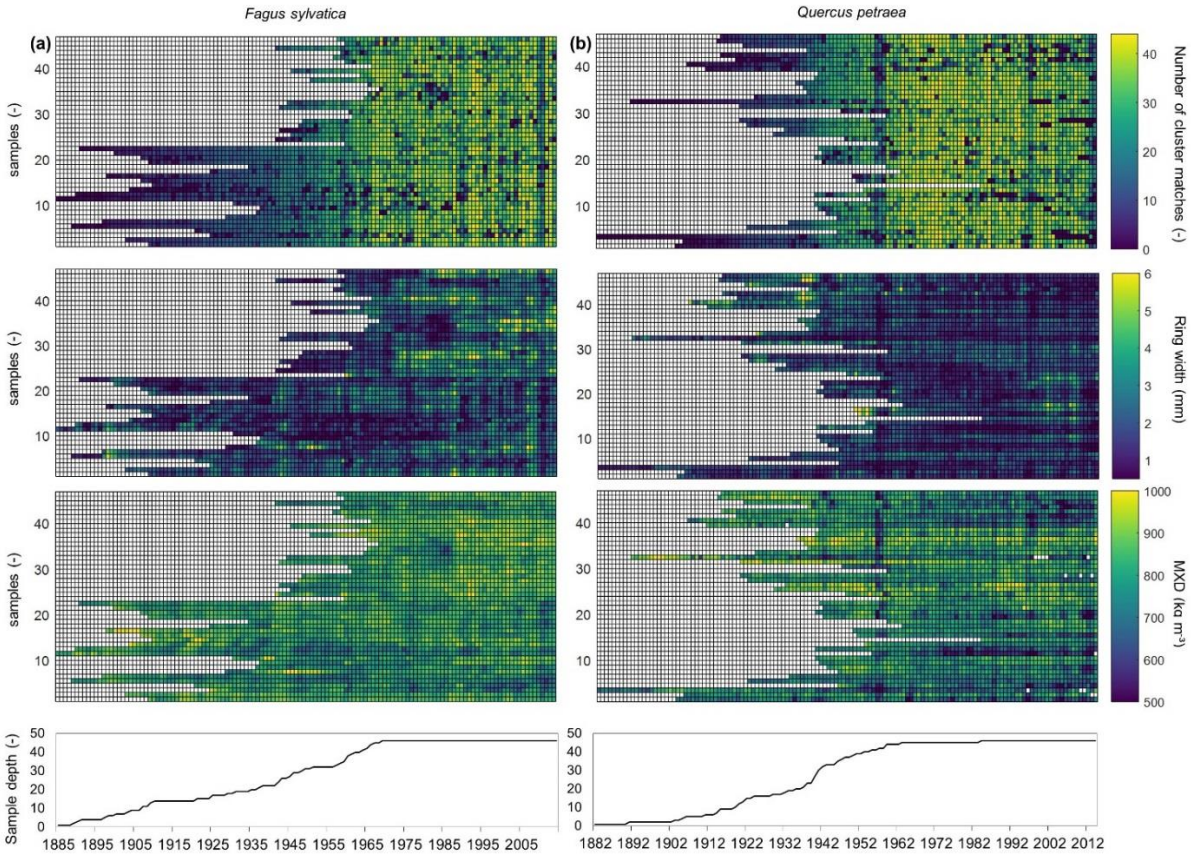

Fig. 4.9 matrix plots of the number of matched years based on density-based pattern matching (DBPM), together with output of ring width, maximum density (MXD) of the cross-dated profiles of (a) Fagus sylvatica and (b) Quercus petraea allow to explore the dataset for patterns.

As a combined output of density and ring width, an envelope profile based on all cross-dated cores is shown for *Q. petraea* and *F. sylvatica* (Fig. 4.10 a,b). The length of each dated intra-annual density segment is rescaled to the average ring width of that year, illustrating density and ring width variability through time.

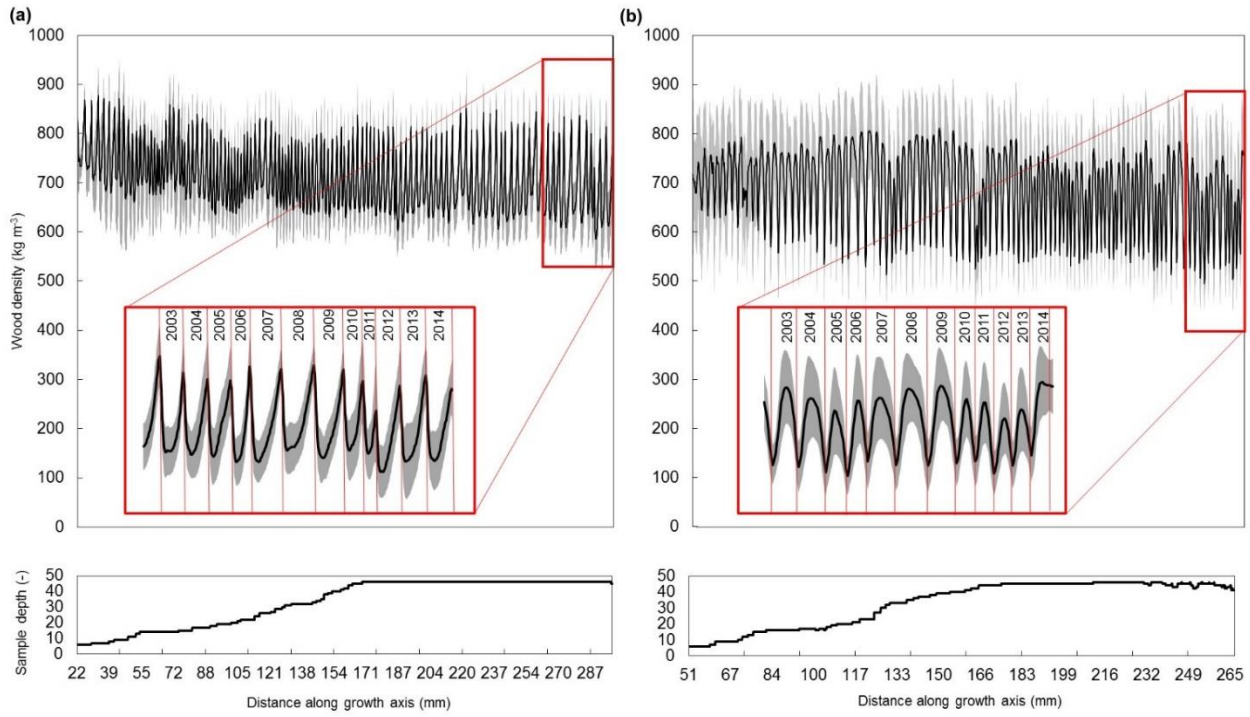

Figure 4.10 Envelope profile of (a) Fagus sylvatica and (b) Quercus petraea profiles visualises ring width and density variation. Error bands show one standard error.

4.4 Discussion

4.4.1 X-ray CT toolchain allows fast screening and archiving

Physical sample manipulation throughout the toolchain remains limited and is considered as a significant advantage in the processing time of large core sample sets, with several steps performed in batch (Supplementary Information Fig. 4.2), resulting in density profiles in a limited time span. The high-throughput character of the toolchain thus meets the need for efficiently measuring variables along large series of rings to increase sample size (Fonti *et al*., 2010). X-ray CT scanning is independent on sample thickness and surface (Van den Bulcke *et al*., 2014), thus 3D-acquisition overcomes several sample preparation steps that occur in classic X-ray densitometry (Polge, 1966; Evans, 1994) as well as high-frequency densitometry (Schinker *et al*., 2003). The resolution of 110 µm used in this study is a result of a trade-off between processing time and accuracy. The required time to perform all batch operations is obviously dependent on CPU/GPU performance of desktop computers. A flexible set-up allows to change resolution in contrast to medical X-ray CT equipment (Steffenrem *et al*., 2014). Sample holders can be tailored to specific needs and

the number of cores depends on the size of the dataset versus the required resolution. Throughout the toolchain, the only manual operation is performed in the GUI, where structure direction and / or rings need to be indicated. Semi-automated tree ring demarcation on the density profiles (Evans, 1994; Mothe *et al*., 1998; Van den Bulcke *et al*., 2014) is implemented, but should always be verified with visual control. 3D corrections are possible, resulting in a re-interpolated density profile that reduces peak flattening (Van den Bulcke et al., 2014). Complicated analysis due to twisting and rolling of the cores (Allen *et al*., 2012) is largely overcome here due to software corrections, since several alignments and structure corrections are implemented. Wood density is directly calculated using a reference material and air, allowing to obtain accurate values (De Ridder *et al*., 2011) and still avoiding the need for calibration functions as in high-frequency densitometry (Wassenberg *et al*., 2014). Consequently, reliable trends of wood density can be pictured for many cores in a fast and efficient way (Fig. 4.5), such as the increasing trend for *T. superba*, which is generally known for light-demanding species (Bastin *et al*., 2015). Extractives and minerals can influence the density profile (Vansteenkiste *et al*., 2007), although for our study species these problems did not occur due to low extractive contents. Species with an extractive content that affects the density profile (Grabner *et al*., 2005) can be extracted with solvents before scanning.

4.4.2 Density-Based Pattern Matching as a suitable parameter for cross-dating

This study is innovative in its goal to use intra-annual density profiles to tackle the challenge of cross-dating, instead of evaluating ring averages of derived density parameters (Allen *et al.,* 2012; Drew *et al.,* 2013). The presented DBPM is a formalization of the classic cross-dating procedure, as the matching relies on higher resolution parameters. The approach of shifts to locate dating errors presented in this paper is comparable to the methodology of shorter segments applied on ring widths in COFECHA (Holmes, 1983; Grissino-Mayer, 2001), yet density profiling allows for shorter series, as more information is available per ring (Fig. 4.6). This study uses Pearson correlation as a similarity measure, but other similarity measures can also be implemented. Although density and ring width matching are difficult to compare, both parameters are complementary in the final evaluation of cross-dating quality, therefore classic ring width parameters are also considered.

It is shown that profiles at 110 µm resolution provide sufficient intra-annual density information for screening the studied samples for cross-dating quality. Moreover, the

nature of the DBPM method implies that significantly small rings not visible on the density profile on this resolution, can be detected by means of DBPM shifting, similarly as anomalies, thus reducing the effect of resolution on the matching (Fig. 4.7). Synchronous density matches (Fig. 4.8) offer scope for further exploration of the dataset. The simultaneous matrix output of density and ring width parameters allows to check for patterns in the dataset (Fig. 4.9).

The limited number of matches for *T. superba* (Fig. 4.8 e,f) is due to a significant growth trend where ring widths tend to drastically decrease at higher age, which complicates matching due to insufficient intra-annual information. Moreover, due to a dominating proportion of vessels and parenchyma, local density variations in these very narrow rings make them less clear toward the bark side (Schongart *et al.,* 2006). The anatomical pattern of ring boundaries formed during senescent growth can be insufficient for reliable demarcation (Stahle, 1999), which is certainly true for *T. superba*, due to its pioneer character and the large number of senescent trees in the dataset. The intrinsic biological nature of the wood structure thus limits the use of the technique (Cherubini *et al.,* 2013). It is unlikely that rings will be better detected at a higher resolution, unless the resolution attains the anatomically resolving threshold, and stem disks should be used to account for wedging rings (Groenendijk *et al.,* 2014). Rejection of large sets of cores is reported in tropical regions (Schöngart *et al.,* 2006), mostly after the time-consuming process of cross-dating. Density-Based Pattern Matching can reduce the effort to discard series before these tedious dating trials on series that are eventually to be discarded. Compared to ring width matching, more series might be withheld based on density parameters (Drew *et al.,* 2013), thus increasing sample size for further analysis of these difficult species.

Many authors do not profoundly discuss the cross-dating process, especially for tropical species, which makes it difficult to assess their work (Wils *et al.,* 2009). The digital X-ray/flatbed image archive together with the ring-indications and profiles presented here ensures that all steps in the cross-dating process can easily be checked and re-assessed. Matrix plots are used for data exploration as they are a powerful tool for visually assessing sample quality. Typical output parameters (e.g. ring width and MXD Fig. 4.9), can be imported in conventional software packages or in other open source tree-ring analysis software (Bunn, 2010) to assess the link with climate. After cross-dating, the user can focus on certain sections containing features such as intra-annual density fluctuations which can, when shown synchronous, be considered as characteristic years. Such years can be spotted and, due to the flexible set-up, processed for detailed anatomical studies with dedicated sample holders. A guided approach of additional scans of particular regions of interest is possible with resolution up to 35 µm (Van den Bulcke *et al.,* 2014) and

eventually to the anatomically resolvable level (Van den Bulcke *et al.,* 2009) (see Chapter 3, Supplementary information Fig. S3.1).

4.5 Conclusion

A non-destructive X-ray CT based screening procedure is presented, going from field to desktop while reducing sample treatment and analysis time. Many research disciplines are focussing on sampling tree cores for densitometry and/or tree-ring analysis, but classic methods are time-consuming, so obtaining data without reducing accuracy is essential. In-house developed software routines treat all data from input (tree cores) to output (dated density profiles). Several batch processes are implemented in the toolchain, minimizing manual effort. Moreover, wood density is determined by rescaling with a reference material.

In contrast to classic X-ray densitometry or high-frequency densitometry, no physical surface treatment is necessary, and cores can be scanned immediately after drying. The digital approach avoids the time needed to surface or microtome the samples, and allows to screen and select large sets of cores. Since many tropical tree species remain poorly studied for their dendrochronological potential, the toolchain allows to screen large sets of cores for such species. It should be noted that not all tropical species might show sufficient density variations at the ring boundaries to be detected with X-CT, which will require surface treatment followed by careful examination of the ring boundaries via classic light microscopy.

High-throughput profiling in combination with exploratory tools such as density-based pattern matching with associated plots are necessary to establish high-resolution chronologies, which is complementary to other techniques that allow to incorporate detailed anatomical, chemical and isotope information (Fonti *et al.,* 2010; Schollaen *et al.,* 2014; Hietz *et al.,* 2015) within tree ring boundaries. The dated density profiles can be further synchronised at the intra-annual level by means of alignment of intra-annual profiles once all cores are cross-dated (Bender *et al.,* 2012).

The toolchain enables cross-dating entirely based on batch generated X-ray CT profiles, which is a new approach in dendrochronology. The obtained similarity, i.e. a correlation based similarity criterion of density information, is a robust and objective method in the decision-making process of cross-dating, which can improve tree-ring analysis for species that are more difficult to match and include more cores in the overall analysis.

This paper aims at filling a methodological gap concerning the integrative approach of tree-ring analysis and more continuous proxies such as density profiles and illustrates how an X-ray CT-based toolchain with minimal labour-input results in a digital archive and data exploration via pattern matching.

5

Cambial activity in the understory of the Mayombe forest, DR Congo

Tom De Mil, Bhély Angoboy Ilondea, Selwin Maginet, Jeroen Duvillier, Joris Van Acker, Hans Beeckman, Jan Van den Bulcke

Abstract

Knowledge on intra-annual xylem growth remains understudied in tropical regions, especially for understory species. However, it is important to disentangle seasonal tree response in this complex environment. The aim is to assess intra-annual wood formation and its variability in selected understory tree species of a semi-deciduous tropical forest.

The cambium of 4 species from the Luki reserve of the Mayombe (DR Congo) was monthly marked at the stem base via the pinning method. To assess ring anomalies on the stem disks, digitization of the last 5-10 rings was performed along the circumference. Relative growth was determined based on X-ray CT volumes of the pinning zone, as well as on sanded surfaces and microsections.

Stem disks allowed to visualize ring anomalies and growth variations. Intra-annual growth was successfully derived via X-ray CT and could be fitted with a Gompertz function. A species-specific response is observed, although there is circumferential variability. However, the most remarkable result is that many of the trees in the dataset had no xylem formation at the stem base, throughout the entire season, thus forming missing rings.

Intra-annual variability in growth illustrates the different responses of species and individual trees to environmental drivers. Phenology might explain the differences, although site and competition should be considered as well. A large number of trees show no xylem growth at all, apart from wound-induced local growth, causing missing rings which has important implications for tree ring analysis in tropical regions.

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Preface Chapter 5

Investigating cambial phenology and leaf phenology in function of positioning intra-annual traits on a time axis, was investigated in Chapter 2. In the following chapter, cambial phenology is explored for understory species of the same forest, that have proven to show a differing flowering pattern (Couralet et al., 2013) and radial growth differences (Couralet et al., 2010).

5.1 Introduction

Studying tree rings in Africa is vital for the assessment of tree response to a changing climate (Gebrekirstos *et al.*, 2014). Due to lower seasonal changes in many tropical regions, tree-ring analysis is often difficult to perform and more information is needed to assess the climate response. Adding intra-annual growth information to these rings leads to specific information which increases our knowledge on how tropical trees react on for instance extreme events such as drought, occurring occasionally within the growth season. Such droughts, for example, can lead to structural changes within the tree ring boundaries, causing intra-annual density fluctuations (Battipaglia *et al.*, 2010, Battipaglia *et al.*, 2013). Despite the importance of revealing intra-annual response of tropical trees, only few studies exist on intra-annual xylem formation (Bauch *et al.*, 2006; Marcati, 2006; Trouet *et al.*, 2012), and especially in the wetter tropics (Amobi, 1973; Dié *et al.*, 2012; Morel *et al.*, 2015). Intra-annual wood growth has been extensively studied on conifers (Camarero *et al.*, 2010; Rathgeber *et al.*, 2011; Cuny *et al.*, 2015) as well as on angiosperm trees (van der Werf *et al.*, 2007; Michelot *et al.*, 2012), through various techniques such as dendrometers (Deslauriers *et al.*, 2003), micro sampling (Rossi *et al.*, 2006, 2013) and cambial pinning, each with their advantages and disadvantages (Mäkinen *et al.*, 2008). Low cost and ease of implementation as well as the possibility of calculating relative growth (Seo *et al.*, 2007), promotes the use of cambial pinnings, especially in remote regions. The cambial pinning technique, first introduced by Wolter (1968), is used to periodically wound the cambium, in order to derive intra-annual wood formation. A thin needle is inserted through the bark and cambium into the xylem of the tree. The wounding initiates the formation of wound tissue and modified wood reaction cells around the pinning canal (Couralet, 2010). The wound reaction can be used as a timer to monitor the amount of wood formation within a certain time period (Seo *et al.*, 2007). Many studies involving pinnings in the tropics are often merely used to check whether rings are formed annually (Mariaux, 1967; Dünisch *et al.*, 2002; Verheyden *et al.*, 2004; Lisi *et al.*, 2008; Chowdhury *et al.*, 2015; Remane & Therrell, 2015), thus not revealing the total intra-annual information within the tree ring. Therefore, intra-annual studies are urgently needed.

However, several challenges occur in revealing the tropical tree response: given the intrinsic plastic behaviour of the cambium in trees growing in regions with less pronounced seasonality (Cherubini *et al.*, 2013), wedging rings and false rings occur and they are mostly described through tree-ring analysis as a cross-dating problem (Worbes, 2002). To our knowledge, this circumferential variability is not being taken into account during cambial pinning experiments (but see (Krepkowski *et al.*, 2012) and (Lupi *et al.,* 2013) for

micro coring), but especially relevant in the tropics. It is also a tedious procedure to prepare proper samples (Mäkinen *et al.*, 2008) and tropical hardwoods have a more complex anatomy compared to conifers, which requires methodological improvements in some cases. Moreover, specifically the evergreen understory and canopy species still remain largely understudied in Africa.

This paper explores intra-annual xylem formation for selected canopy and understory trees of the Luki Biosphere Reserve. We hereby complement previous research in the study area (Couralet *et al.*, 2010), which has shown species-specific response based on dendrochronology and seasonal diameter measurements. X-ray CT imaging was proposed as a method to infer intra-annual xylem growth; it was shown before that X-ray CT can be used to assess the wound tissue non-destructively in 3D (Stoffel & Klinkmüller, 2013, Ballesteros-Cánovas *et al.*, 2015). In this study, the primary objective was to explore the variability of intra-annual xylem formation between species, between individuals and finally at the individual level.

5.2 Materials and Methods

5.2.1 Study site

The selected individuals all grew in the same old-growth forest stand, close to the INERA research station of Luki. Species were selected based on their abundancy, ability to form growth rings (Fouarge & Gérard, 1964; Couralet *et al.*, 2010) and degree of wedging, based on previous sample campaigns (data not shown). Selection criteria in the field were health, maturity, and, if possible, roundness and straightness of the stem.

5.2.2 Cambial marking and sample preparation

Twelve trees of 4 different species (3 *Greenwayodendron suaveolens*, 3 *Xylopia wilwerthii*, 3 *Corynanthe paniculata* and 3 *Aidia ochroleuca*) in the same site were selected and pinned monthly with a straight pin during one growth season (11 months, starting from 21/09/2013 till 21/07/2014). Each monthly pinning was repeated three times in a spiral arrangement (Figure 5.1), shifted 5 cm to one another to avoid interference of nearby pinnings. The selected species are evergreen mesophanerophytes (8-30 m in height),

except for *G. suaveolens,* which is a megaphanerophyt (more than 30 m in height). All species are widespread in the Guineo-Congolean forest domain.

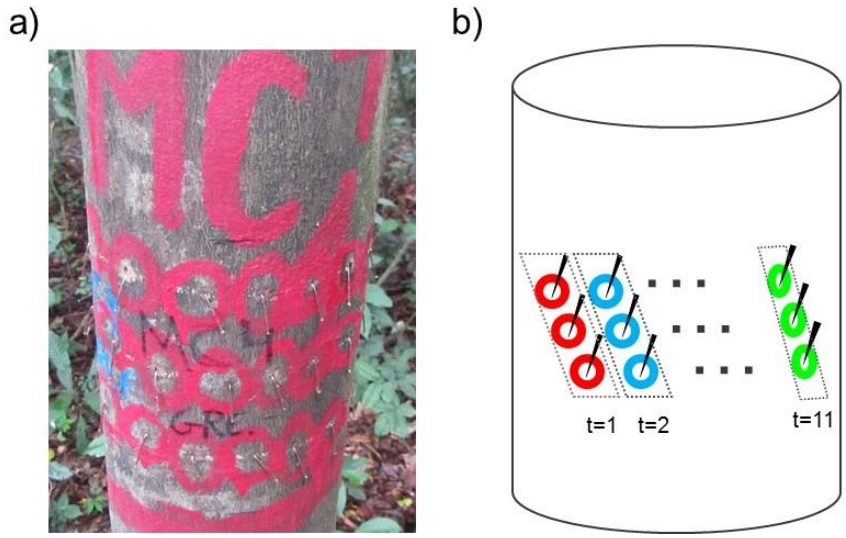

Figure 5.1 G. suaveolens individual with a) monthly cambial pinnings and b) corresponding sample scheme of pinnings and replicates.

In September 2014, all trees were felled and stem discs including the pinnings were sampled. Subsequently, stem disks were dried at room temperature (about 25°C) during a period of 4 months after which discs were subdivided into 3 smaller disks each containing 11 consecutive monthly pinnings. Additionally, pinning samples for *A. ochroleuca*, *C. paniculat*a and *X. wilwerthii*, were available from a previous pinning campaign in the season 2006-2007, from a site nearby the same study area (Couralet *et al.*, 2010).

5.2.3 Imaging

The stem disk surface was progressively sanded (50-600 grid size) for proper visualization of the macroscopic tree ring structure. The pinned stem disks were scanned with a flatbed scanner (EPSON Perfection 4990 PHOTO), at a resolution of 1200dpi, followed by the digitization of the last 5-10 rings with ImageJ (Schneider *et al.*, 2012) in order to identify ring wedging. These rings can serve as an overlay and enable the detection of anomalies before sampling of the pinnings for further analysis.

Cylinders of approximately 1.5 cm in diameter and 1 cm in length containing the pinnings, were then sampled from the stem disks. These cylinders were scanned with X-ray CT,

resulting in a 3D view on the wood structure around the pinned zone. This allowed to assess the pinning structure and quality before sanding or cross sectioning.

The Nanowood scanner used at Woodlab-UGent is a setup developed at UGCT (Dierick *et al.*, 2014), the Ghent University Centre for X-ray Tomography, in collaboration with XRE (www.XRE.be). Specimens were scanned with standard cone-beam X-ray CT with a scan time of approximately 21 min per sample. Reconstruction was performed with Octopus, a tomography reconstruction package for parallel, cone-beam and helical geometry as well as phase correction and retrieval (Vlassenbroeck *et al.* 2007, licensed by InsideMatters: www.insidematters.eu). Beam hardening correction was applied, both by hardware as well as software filtering. The obtained approximate voxel pitch is 10 µm.

Anatomical differences between regular wood structures and wound-induced features enable the localisation of the cell boundary before and after pinning of the cambium. X-ray CT guided thin cross-sections of pinning sections were taken for detailed study of the wound tissue if relative growth could not be measured on the X-ray CT volumes.

5.2.4 Intra-annual xylem formation

The increment from the beginning of the growth season up to the pinning date was measured. Following Seo *et al.* (2007) the increment up to the harvesting date was additionally measured to correct for eccentric xylem growth. The measurements provide a time series of relative radial growth during almost one growth season.

A Gompertz function (Gompertz, 1825 in Winsor, 1932) was fitted to the time series of relative growth data. The Gompertz function has been applied in several studies of radial xylem growth in boreal and temperate regions (Deslauriers *et al.*, 2003; Rossi *et al.*, 2003; Mäkinen *et al.*, 2008), but applications for tropical regions are not reported. Because of its flexibility and asymmetrical shape, the Gompertz equation has been proven successful to describe growth-time relationships (Zeide, 1993; Mäkinen *et al.*, 2008). The Gompertz equation is defined as:

$$RG = \mathrm{a}\ \mathrm{e}^{-\mathrm{e}^{\beta-\kappa t}}$$

Where RG is relative growth, a is the upper asymptote, β is the x-axis placement for the location of the origin (Cuny *et al.*, 2013), κ stands for the rate of change parameter and t

is the time expressed as the number of days from the first cambial pinning (here, 21/09/2013 and 01/10/2006 for the considered seasons). All processing steps were done in Matlab R2015b®.

The cylinder samples were cut to cubes of about 1 cm^3 containing the pinning. These cubes were heated in the oven for 24 h-48 h at 70 °C in a mixture of 60 % H_2O, 30 % glycerol and 10 % alcohol to soften the tissue for proper micro sectioning. Transversal sections with a thickness of 20-30 µm were cut on a sliding microtome and coloured with safranin/ astra blue and fixed in Euparal resin.

5.3 Results

An overview of the sampled trees with their biometric parameters is given in Table 5.1. Some pinnings could not be used because the needle did not reach the cambium due to the thick bark.

Table 5.1 Overview of the studied samples

	Species	N° of trees	Tree height range (m)	DBH range (cm)	Digitized disks	X-ray imaging	Intra-annual curve	Zero growth
2013-2014	*Greenwayodendron suaveolens*	3	20-25	22.9-34.5	3	3	3	-
	Aidia ochroleuca	3	12-14	22.6-26.3	3	3	3	-
	Corynanthe paniculata	3	12-22	12.4-27.5	3	3	1	2
	Xylopia wilwerthii	3	11-12	18-29.3	3	3	3	-
2006-2007	*Aidia ochroleuca*	4	-	14.9-23.0	-	-	4	-
	Corynanthe paniculata	7	-	18.4-46.0	-	-	5	2
	Xylopia wilwerthii	6	-	15.4-21.0	-	-	-	6

5.3.1 Digitized ring contours and X-ray CT based relative growth measurements

The digitization of stem disks and ring indications allows mapping of irregular stem growth and assessing the impact of wedging (Fig. 5.2). Thus, wedging rings and eccentric growth can easily be detected. Furthermore, this allows to locate the pinnings accurately on the digitized disks when subsampled for X-ray CT.

Using X-ray CT imaging, one is able to section the pinning virtually, at any direction to search for the best cross-section to perform the measurements (Fig. 5.3). The axial region

to perform the distance measurement is not always right on the pinning spot, but rather more above and below the wounding. Bright spots around the pinning zone are due to iron oxide of the needle as well as wound-induced depositions. Xylem growth is visible after the wound formation, thus distances can easily be measured (arrows Fig. 5.3).

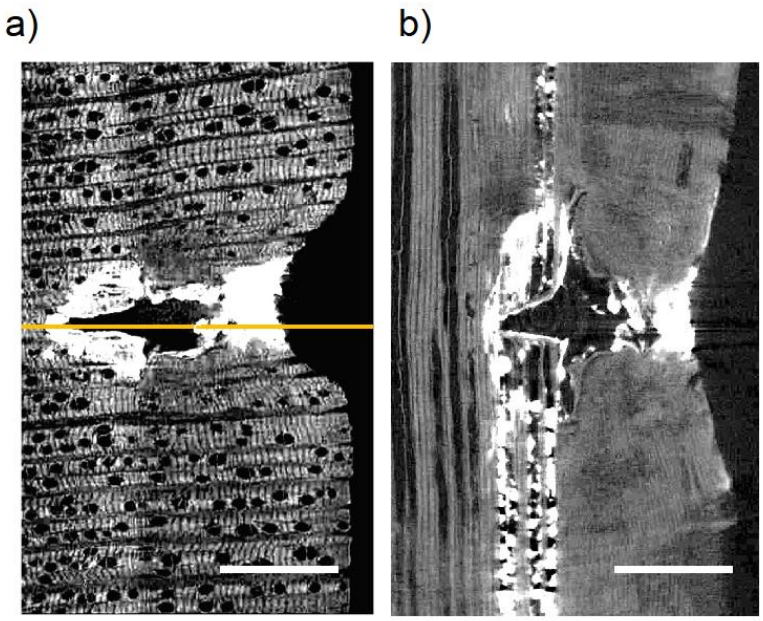

Figure 5.2 X-ray CT images allow to sample the best cross-section for relative growth measurements. a) transversal view with a section (horizontal line) of the b) radial view. Scale bar = 1 mm.

5.3.2 Species-specific xylem formation

All species in this study show xylem growth cessation during the dry season: on the X-ray CT pinning images of July only the needle insertion is visible and the tree ring has fully developed (Fig. 5.3). A species-specific xylem growth response is observed and Gompertz functions could be fitted to the relative growth increments (Fig. 5.4, Table 5.2). The peak of xylem growth for all individuals seems to be more or less synchronous, around February-March. All individuals of *A. ochroleuca* showed a similar intra-annual growth pattern, starting between September and October 2013 and ending between May and June 2014 (Fig.5.4 a).

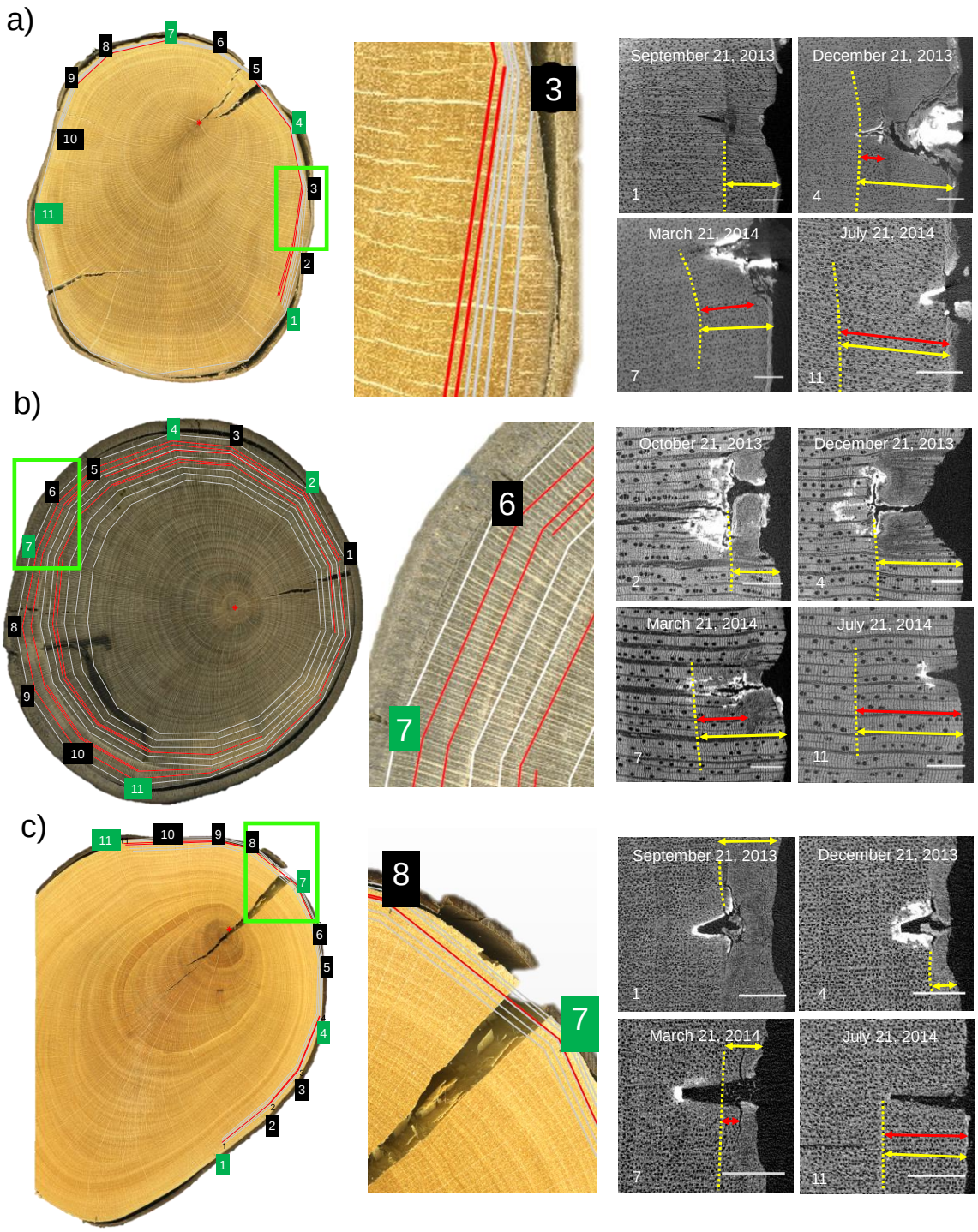

Figure 5.3 Stem disk images with digitized contours of the last 5-10 rings including wedging rings, derived from digitized stem disks for a) A. ochroleuca, b) G. suaveolens and c) C. paniculata. White lines represent normal rings, red lines indicate wedging rings. For a) and c) measurements were only performed on a section of the total circumference. Indication of the measurements on the X-ray cross section is shown: dashed line is the tree ring boundary, red arrows indicate the growth till pinning, yellow the total ring width to achieve relative growth. Scale 1 mm.

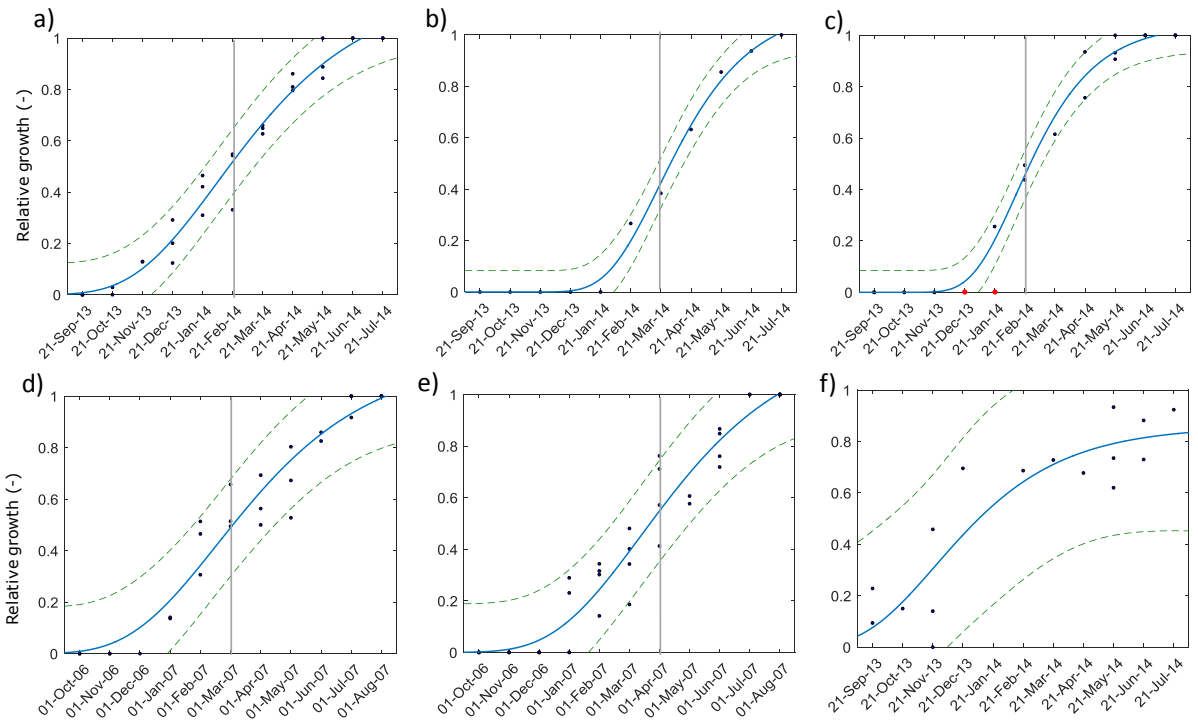

Figure 5.4 Intra-annual relative growth for a) A. ochroleuca, b) C. paniculata , c) G. suaveolens and f) X. wilwerthii for the growth season 2013-2014. For d) A. ochroleuca and e) C. paniculata growth curves of the season 2006-2007 were available as well. Solid lines represent the Gompertz fit, dashed lines the 95% prediction interval. Vertical lines indicate the point of inflection, i.e. the maximum growth rate.

Table 5.2 Statistical parameters of the Gompertz fit.

Species	Year	A (95% PI)	B (95% PI)	C (95% PI)	SSE	R square	DFE	Adj R-sq	RMSE
A. ochroleuca	2013-2014	1.20 (1.03, 1.36)	1.98 (1.58, 2.38)	0.36 (0.26, 0.46)	0.10	0.98	27	0.97	0.06
	2006-2007	1.12 (0.93, 1.30)	2.08(1.48, 2.67)	0.38(0.25, 0.51)	0.20	0.96	30	0.96	0.82
G. suaveolens	2013-2014	1.04 (0.989, 1.10)	3.88 (3.09, 4.67)	0.68 (0.54, 0.82)	0.04	0.99	21	0.99	0.04
C. paniculata	2013-2014	1.11 (0.96, 1.26)	4.04 (2.88, 5.20)	0.58 (0.40 0.77)	0.01	0.99	8	0.99	0.04
	2006-2007	1.23(0.94, 1.51)	2.23 (1.60, 2.85)	0.35 (0.21, 0.49)	0.30	0.94	35	0.94	0.09
X. wilwerthii	2013-2014	0.86 (0.59, 1.13)	1.31 (0.08, 2.54	0.43 (-0.05, 0.90)	0.32	0.79	13	0.76	0.16
	2006-2007	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

PI prediction interval, SSE Sum of Squared Errors of Prediction, R square Coefficient of determination, DFE Degrees of Freedom for Error, Adj R-square Adjusted Coefficient of determination, RMSE Root-Mean-Square Error

Three individuals of *G. suaveolens* also showed similar intra-annual wood formation, starting between December and January (Fig. 5.4 c). Despite some wedging in the previous rings, a complete ring was formed during 2013-2014 (Fig. 5.3 b). Yet, one *G. suaveolens* individual had clear wedging with pinning 4, 5, 6 and 7 occurring on an absent ring (Fig. 5.4 c). Stem disks, fortunately, allowed to interpret this wedging. The remainder of the pinnings on this individual were included for the Gompertz fitting.

We could only establish a growth curve of one *C. paniculata* individual (Fig. 5.4 b). This individual only starts growing in January and has a substantial growth lag compared with the other species in 2013-2014.

For *A. ochroleuca*, the growing range of 2006-2007 is of the same order as observed in the season 2013-2014 (Fig. 5.4 a, d). However, growth of *C. paniculata* in 2006-2007 started growing much earlier compared to the season 2013-2014 (Fig. 5.4 b, e), which is similar to the curve of *A. ochroleuca* (Fig. 5.4 b vs. Fig. 5.4 e).

5.3.3 Intra-individual growth: ring anomalies

In addition to wedging for the *G. suaveolens* sample, more extreme cases of ring anomalies occur: two *C. paniculata* individuals even have no discernible growth, while only wound-induced tissue is observed (Table 5.1, Fig. 5.5 a, Fig. 5.6), resulting in a missing ring. The extent of the wound-induced xylem clearly shows that the tree still has a living cambium, despite not forming any detectable xylem growth elsewhere on the stem disk.

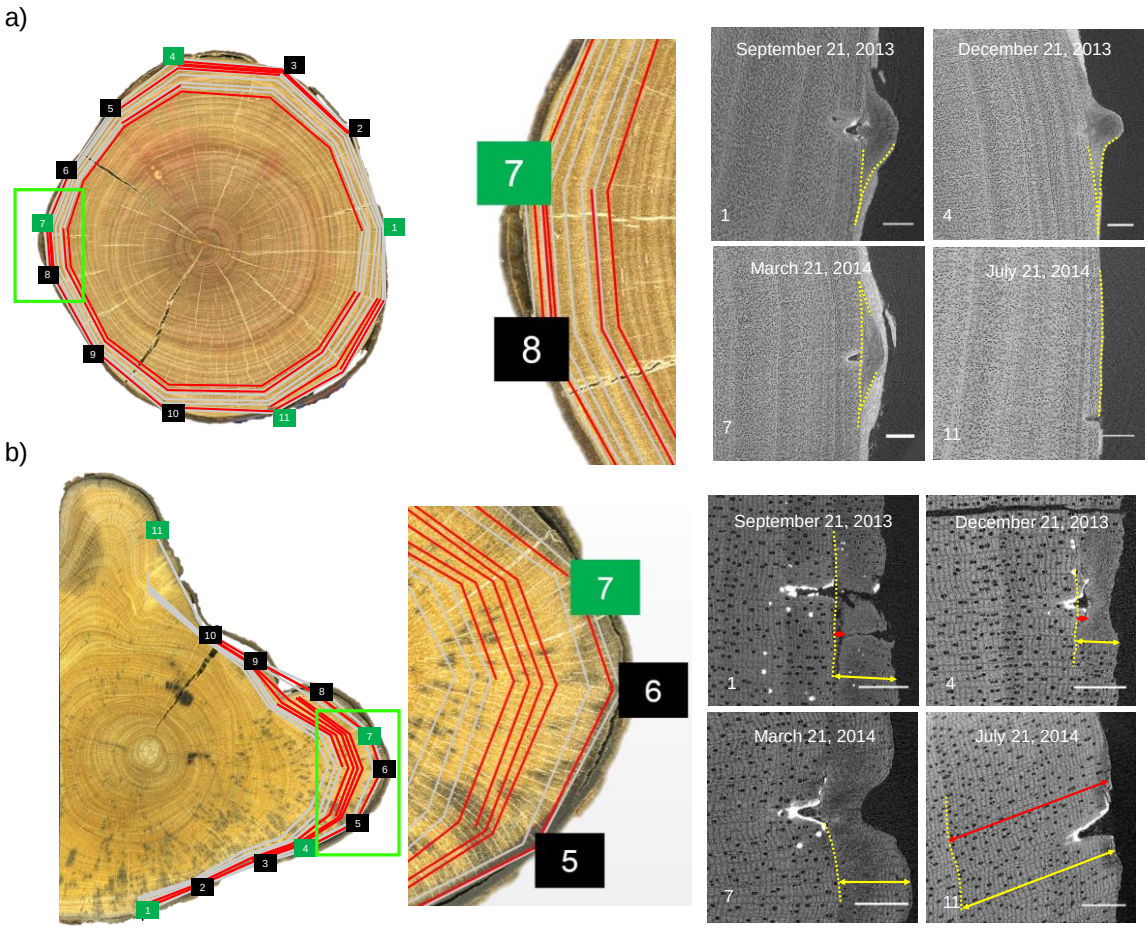

Figure 5.5 Stem disk images with digitized contours of the last 5-10 rings for a) C. paniculata showing only wound-induced xylem growth, and b) X. wilwerthii showing circumferentially variable intra-annual growth. White lines represent normal rings, red lines indicate wedging rings. For b) measurements were only performed on a section of the total circumference. Scale bar = 1 mm.

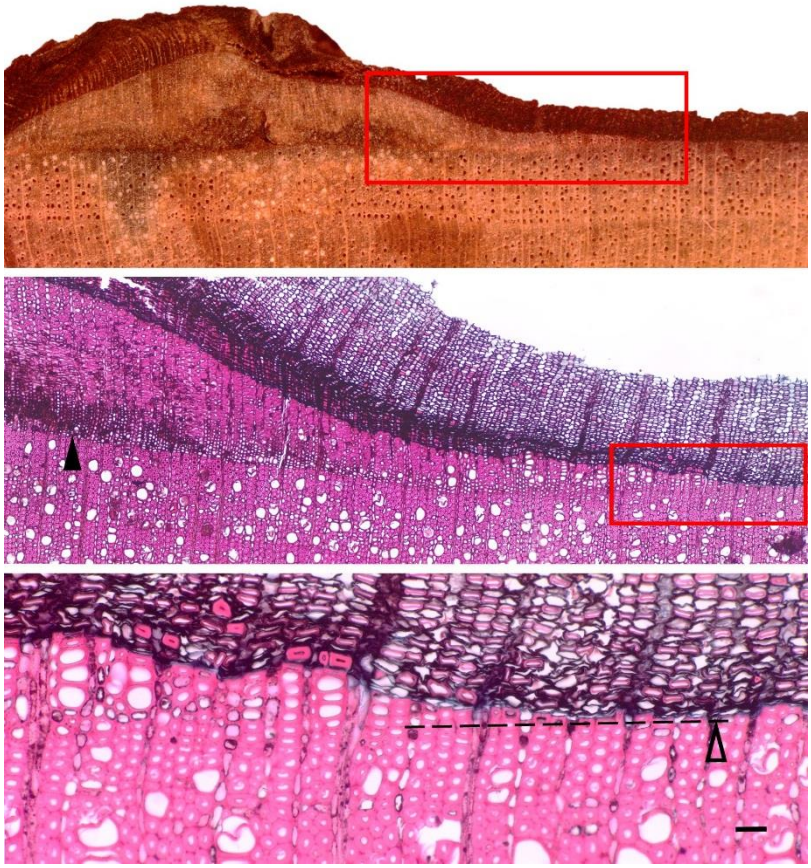

Figure 5.6 C. paniculata sample with a pinning from September 2013. Only pinning-induced local growth occurred for the entire growth season, which means no ring was formed. Full arrow indicates callus formation above the pinning wound, empty arrow shows onset of wound-induced xylem, dashed line indicates previous growth ring boundary. Scale bar = 10 µm.

Next to wedging, significant circumferential variability hampers proper analysis of cambial pinnings for the *X. wilwerthii* samples from 2013-2014. This variability is seen on the digitized stem disks as highly variable ring width (Fig. 5.5 b).

Although radial growth could be measured, the circumferential variability in intra-annual growth rate hampered the construction of a consistent intra-annual curve (Fig.5.4 f), even if relative growth was always present (X-ray images Fig. 5.5. b). Fitting of a Gompertz model was possible, but model quality parameters are considerably lower (Table 5.2).

5.4 Discussion

5.4.1 Ring digitization to account for anomalies and X-ray CT for measuring growth

The digitization of stem disks allows to assess the circumferential variability on the stem disks, similar to the annual ring diagrams described in Larson (1994). It is a convenient way to identify matching rings and anomalies along the entire circumference, and is of high importance for trees in tropical regions (Latte *et al.*, 2015). X-ray CT allows to assess the wound tissue in 3D, before sanding or sectioning, which might erase the pinning spot and could lead to sample loss.

5.4.2 Species-specific and intra-species variation in intra-annual response

The early onset of the *A. ochroleuca* data concur with earlier investigations based on monthly diameter measurements (Couralet *et al.,* 2010). The most significant observation for 2013-2014 is the late onset of growth for *G. suaveolens* and *C. paniculata*. For *G. suaveolens* this observation is consistent for the 3 individuals, whereas for *C. paniculata* only 1 individual has a late onset of growth, while the other two individuals only show wound-induced cambial activity during the entire growth season. Some wedging was observed for *G. suaveolens*, but for all individuals a growth curve could be established, as this species is known to have dendrochronological potential (Worbes *et al.*, 2003). For *C. paniculata*, the observed late onset concurs with Couralet *et al.* (2010), based on tree-ring analysis, where the species reacts on late-season climatic events.

Combined observations of phenology and wood formation are key in understanding tree growth response in temperate regions (Michelot *et al.*, 2012). Although no direct connection between flowering and wood formation has been found yet in the tropics, phenology monitoring of these evergreen species could tell whether leaf and cambium phenology are synchronous. A forest-wide bimodal flowering pattern based on data from Couralet *et al.* (2013) could explain the differing growing onsets. For *A. ochroleuca* and *G. suaveolens*, the respectively early and late onset coincide with the flowering pattern seen in Fig. 5.7. This bimodality concurs with findings of Borchert *et al.* (2005).

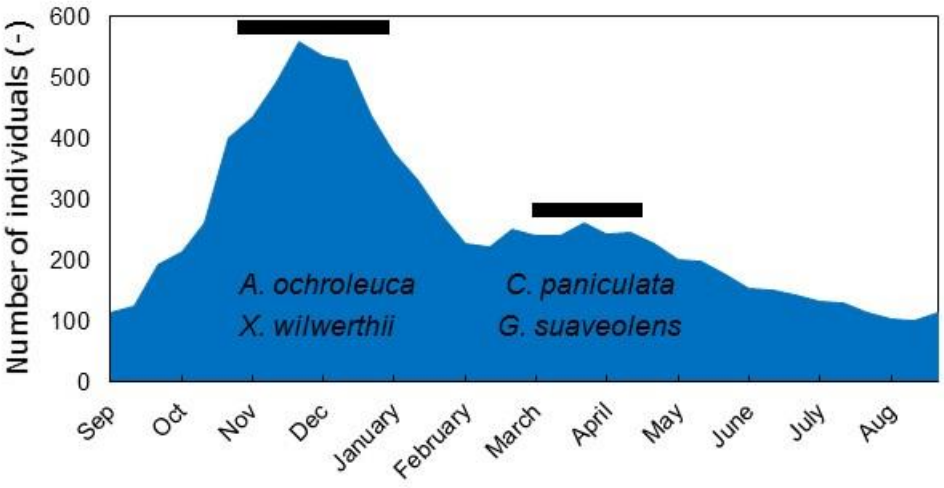

Figure 5.7 Forest-wide bimodal flowering pattern of the Luki reserve, data adopted from Couralet et al. (2013)

However, there are large differences in onsets between the season 2013-2014 and 2006-2007 for *C. paniculata.* Naturally, these trees were sampled from a different site and a different season. Thus, other factors such as site as well as social status of the individual due to competition can also have a considerable influence on the onset (Rathgeber *et al.*, 2011). Increasing competition can lead to later onsets and smaller ring width. Ultimately, a further lack of assimilates due to this competition will hamper proper ring development along the entire circumference, which leads to intra-individual variations.

5.4.3 Intra-individual variation: wedging and zero xylem growth

The studied understory species show different ring anomalies, ranging from wedging to the extreme case of zero growth, resulting in missing rings and concurring with the zero radial increment of certain individuals observed by Couralet *et al.* (2010) at the sampled height.

Unfortunately, little information exists on the causes of these ring anomalies and their drivers in the tropics. The only literature at hand comes from temperate and boreal regions, with trees growing under climatic stress. The understory position of the trees in this study, however, also gives rise to stresses such as changing light conditions (Worbes & Fichtler, 2010) and a small crown due to competition, thus provoking limited availability of assimilates which could be the cause of an increased wedging. Indeed, Wilmking *et al.* (2012) mention that tree species which are limited in resources start forming wedging

rings, and consecutively higher stress levels cause zero xylem growth at the stem base resulting in missing rings. Using digitized stem disks, we indeed noticed that multiple wedging rings occur in growth years prior to the pinning season, which could indicate an increase in stress eventually provoking zero growth of the *C. paniculata* samples from 2013-2014. Finally, it is known that missing rings are more frequent in older and larger trees (Novak *et al.*, 2016), but no direct link could be found here between tree size and frequency of wedging for the samples investigated (Table 5.1).It should be mentioned, however, that tree-ring studies along the stem axis (Fritts, 1976; Leland *et al.*, 2016), as well as vertically consecutive tree diagrams (Larson, 1994) show that tree rings can still form further upwards in the canopy, even though missing rings are present at the sampled height.

Our observation of wound-induced xylem formation adjacent to the needle insertion (Fig. 5.6), despite the zero growth elsewhere along the circumference, gives indirect evidence of ring formation further upwards in the stem axis. The phloem fails to provide assimilates for actual xylem formation at the stem basis, but is still able to form wound-induced xylem. This could imply that the cambium is inactive but can be activated by wounding, resulting in local growth (Copini *et al.*, 2014), a term which we will refer to instead of wound-induced xylem growth. In the latter study, local growth was measurable at the end of the growth season while being masked earlier in the season. However, in our study the zero growth samples show local growth all year round. What is even more, in some cases wood formation does not occur around the wound-induced zone yet the ring tends to reappear further away from the pinning along the circumference. Hypothetically speaking, when resources are limited, a local triggering of a hormonal response (Copini *et al.*, 2014) could lead to a compensational mechanism with a local decrease in xylem growth in the area adjacent to the local growth zone.

When excessive circumferential variability in growth rate exists, however, such as for *X. wilwerthii* (Fig. 5.4 f, Fig. 5.5 b), accurate intra-annual xylogenesis curves cannot be obtained. Abundant wedging occurs in the previous rings, but not during the pinning experiment. Despite corrections applied here, as reported in Seo *et al.* (2007), the intra-annual growth seems to be too irregular, making it difficult to see in what part of the season *X. wilwerthii* is growing, emphasizing the remarkable asymmetrical plasticity of the cambium around the circumference. This is a major challenge in tree-ring analysis (Cherubini *et al.* 2013) as eccentric stems might exhibit circumferentially differing tree response (Buras & Wilmking, 2014).

5.5 Conclusion

This is the first study of intra-annual xylem growth on understory species in tropical Africa. Stem disks were digitized to map and track ring anomalies such as wedging. X-ray Computed Tomography proves to be an efficient tool for measuring relative growth, and if needed, provides a guided selection for micro sectioning or sanding. We show varying intra-annual growth patterns, which implies species-specific responses on climate. The bimodal flowering pattern, might be an explanation for the intra-annual growth differences between species. However, these responses can also be influenced by other factors such as site and competition. As seasonality is weaker in the tropics compared to more temperate regions, the impact of aforementioned factors can be considerable. The striking observation that so many sampled pinnings showed no xylem growth at the stem base, apart from wound-induced local growth, emphasizes the complexity of tropical wood formation and resource availability of these species. There is a need for additional thorough research on the phenomena such as local growth and wound-induced wood formation. The aspect of wedging and missing rings is well documented in temperate and arctic regions. However, it is rarely studied in the tropics, though it is being widely acknowledged as a problem in tropical tree-ring analysis. Therefore, pinning and related studies are essential to explain difficulties in tree ring analyses, especially in the African forests, where many species remain understudied.

6

Nailing the facts: 66 years of individual radial tree growth in Central Africa

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Abstract

Multi-decadal tree growth observations are scarce in Central-African forests and are vital in understanding tree dynamics. Permanent sample plot data cover maximally a few decades, and studies on tree age and growth patterns of rainforest trees often face dating. This study therefore combines archived diameter measurements with analysis of dated nail marks in the xylem in order to link radial increment to xylem increment by use of a unique set of rediscovered trees.

In this study, records of diameter data of 4767 trees covering 176 species measured from 1948 to 1957, were digitized and analysed. Based on this dataset as well as archived maps, 414 trees were retrieved in 2014. The diameter of the rediscovered trees was measured and the nails carrying the original 1948 number tag, serve as a cambial timestamp in the wood. 134 samples from 18 species were taken containing the nail marks in order to analyse radial xylem growth.

Diameter growth is higher in 1948-1957 than in 1957-2014. A shift in fast growers is seen, mainly from pioneers contributing to the radial growth in 1948-1957, to canopy species in 2014.The most striking observation is that many species do show diameter growth after 66 years, but only form 32 rings on average, thus forming non-periodical rings, indistinct rings or no xylem formation for multiple decades.

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Preface Chapter 6

In Chapter 4 we have established a toolchain that is suitable for high-throughput processing of increment cores. Specifically, Density-Based Pattern Matching has proven to be useful to crossdate well known temperate species F. sylvatica and Q. petraea, and medium-sized to large rings of T. superba. The next chapter uses that toolchain on other species of the Mayombe from different functional groups, including the understory species of Chapter 5, that are part of a unique long-term monitoring experiment area at the Nkulapark.

6.1 Introduction

Knowledge on age and long term radial growth rates in tropical trees remains scarce (Clark, 2007; Worbes *et al.*, 2003), but is important to accurately predict carbon storage in a globally changing environment (Lewis *et al.*, 2009).

Therefore, permanent sampling plots are of substantial interest. Diameter measurements based on census data encompass all species in the forest in order to draw conclusions on forest-wide biomass increment (Brienen *et al.*, 2015). In Africa, temporally and spatially distributed measurements (Lewis *et al.,* 2009) are increasingly being conducted. Yet, the only reported long term forestry plots in Africa spanning more than 5 decades are mentioned in Sheil (1995) and Phillips *et al.* (1998). Other dating methods such as radiocarbon dating (^{14}C) can be used as well (Chambers *et al.*, 1998; Vieira *et al.*, 2005) and it has been proven that tropical trees can reach ages of more than 1000 years.

However, ^{14}C analysis is limited in use for trees, as being only accurate before 1650 and after the 1960's (Worbes & Junk, 1999) and a more direct way of dating trees is needed, such as by investigation of the xylem structure. The xylem contains traits functioning as descriptors of the past and allows to perform tree-ring analysis, as such reaching further back in time than permanent sample plots whilst obtaining a higher level of detail of radial growth (Rozendaal & Zuidema, 2011). Several tropical trees that have discernible rings (Worbes, 2002), are instrumental for dendroclimatology (Schöngart *et al.*, 2006; De Ridder *et al.*, 2013), as well as for tree ecology and demography studies (Zuidema *et al.*, 2010). Although research is ongoing to assess the tree ring potential of lesser studied species (Groenendijk *et al.,* 2014), often, the selection of species for dendrochronology does not concur with those of census data. Sampling strategies for tropical tree-ring analysis traditionally include upper story, medium-to-fast growing species of economic value in order to obtain information on yield or sustainable management purposes (Stahle *et al.*, 1999, De Ridder *et al.*, 2013; Groenendijk *et al.*, 2014).

Furthermore, many species do not form anatomically discernible rings (Tarelkin *et al.*, 2016), and for several species the annual character of the ring pattern remains ambiguous due to wedging, double and missing growth rings (Dié *et al.*, 2012). Therefore, there is a clear need of having an accurate timestamp of wood formation.

One way of establishing this, is via tree-ring analysis of planted tropical forests with known age. Unfortunately, well documented planted forests are still too young to be properly assessed and have problems with dating the juvenile stage (De Ridder, 2011). Moreover, the conditions of plantations are not the same as those from natural forest. Cambial wounding is another method for dating the wood structure (Mariaux, 1967; Detienne, 1989; Lisi *et al.*, 2008), but these experiments often lack long term assessment of the aforementioned anomalies.

Tree rings of the understory (Couralet *et al.*, 2010) and canopy trees are less studied, despite the high stem density (trees ha^{-1}) of understorey trees. Competition of canopy and emergent trees, which limit climate sensitivity of the trees, and thus, its ability to form annual rings, often has hampered proper tree-ring analysis. Given the complex tropical forest composition, it is important to assess the radial xylem growth rate and response of all trees in function of a changing climate, rather than the few species suitable for classic tree ring analysis. More specifically in Africa, there are large knowledge gaps on how to derive information from the past through tree rings (Gebrekirstos *et al.*, 2014), not only from understory, but from all functional groups (Lieberman *et al.*, 1985), due to a large number of understudied species.

Here, we present a forest-wide assessment of long term tree growth in an old forestry plot in the Mayombe forest (DR Congo). This study exploits the unique availability of (i) well documented cambial marks in the wood from 1948 combined with (ii) archived (1948-1957) and recent (2014) diameter measurements, in order to analyse periodical radial increment via Diameter measurements at Breast Height (DBH), with annually resolved xylem increment. Individual rates of 66 years of radial growth are assessed and compared with the archived growth layers in the xylem.

6.2 Material & Methods

The Nkulapark (174 ha) is a phenology (Couralet *et al.*, 2013) and tree growth monitoring park (Fig. 6.1) established in 1947 and managed by the INERA (http://www.inera-drc.org/accueil) within the Luki Biosphere reserve (Fig. 6.1 a,b). From 1948 to 1957 yearly diameter measurements were performed on trees covering the entire area of the park along 29 km of pathways following the relief contours (Fig. 6.1c). This park consists of (i) natural stands of *Prioria balsamifera* and (ii) *Terminalia superba,* (iii) secondary forests in the valley with characterizing herbal vegetation of *Zingiberaceae* and *Marantaceae*, (iv)

savanna that is being colonized by forest, (v) modified forests (fôrets remaniées) and (vi) old-growth forest on the East of the Nkula brook (INEAC 1947, ARA Nilco 2014). The measurements were suspended in 1957. Tree selection encompassed all diameter classes and species.

The area is situated almost entirely in a catchment with a valley and a ridge in order to include several microclimatic conditions. In this study, the repeated diameter measurements of 6300 trees were digitized. After data cleaning of the diameter data (Talbot *et al.*, 2014; Brienen *et al.*, 2015), negative growth not smaller than 5mm, and positive growth not exceeding 40mm, 4767 trees were retained for further analysis.

6.2.1 Retrieval of trees

Each of the trees were labeled in 1947-1948 with a number tag that was attached to the tree using 8 cm long nails. A field campaign in 2013-2014 resulted in the retrieval of 400 out of the original set of 6300 individuals. The search was based on an original old map that was digitized and georeferenced with QGIS (QGIS development team, 2016) using specific landmarks (paths, specific trees, etc.). The old observation paths were located and trees along these paths with tag (Fig. 6.2a) or scars on the trunk indicating an overgrown nail and repelled number tag on or in the ground (Fig. 6.2b) were tracked. Half of all retrieved trees were found using a metal detector (BHJS, Bounty Hunter, USA) by scanning a) the trunk for presence of nail traces followed by scanning the b) soil adjacent to the tree to retrieve repelled number tags. Nails down to 15-20 cm underneath the bark surface could successfully be tracked. The retrieved number tags were in most cases still readable, and could be related to the digitized dataset of 6300 trees. Nails were remarkably preserved, probably due to cathodic protection of the zinc number tags (Supplementary Material Fig. S6.1). Trees were measured at the same height as the original diameter measurements, often indicated by paint which was often still visible adjacent to the number tag. Forked trees were not considered for further analysis.

6.2.2 Tree sampling

The nail marks in the wood are actually cambial pinnings and thus provide us with unique information on the individual 66-year xylem response of these trees. The distance between the nail wound reaction and the cambium can be measured in order to obtain total xylem

growth (Fig. 6.2.). Therefore, from a subset of 134 trees (Table 6.1) increment cores were taken containing a nail mark serving as an absolute timer. Cores were scanned at 110 µm resolution (Fig. 6.3) with the NanoWood CT facility (Dierick *et al.*, 2014), developed in collaboration with XRE (www.XRE.be). Reconstruction was performed (GPU GeForce GPX 770 4GB) with the Octopus reconstruction software package (Dierick *et al.*, 2004; Vlassenbroeck *et al.*, 2007; licensed by InsideMatters: www.insidematters.eu). Distance between the cambial mark and the cambium was measured with imageJ (Schneider *et al.*, 2012) and tree rings were counted using the X-ray CT-based tool-chain for tree-ring analysis described in De Mil *et al.* (2016). From a selection of trees, stem sections were sampled including the nail mark, to have a clear view on the wound tissue. The distance between nail mark and cambium, as well as tree ring width, were measured with a Lintab measuring system (Rinntech, Germany) and a stereomicroscope (SZX 12, Olympus). Two additional cores were taken at 120° from the nail trace along the circumference (Fig. 6.2b). When this resolution seemed insufficient, the cores were surfaced with the core microtome (Gärtner & Nievergelt, 2010) and the surface was imaged using a flatbed scanner (EPSON Perfection 4990 PHOTO) and the distance between the 1948' cambial mark and the cambium was measured with imageJ.

Table 6.1 Available datasets of the Nkulapark

Data	N° of trees	N° of tree species
10 y of radial growth (1948-1957)	4767	176
57 y of radial growth (1948-2014)	414	87
Cores and stem sections with cambial mark from 1948'	134	18

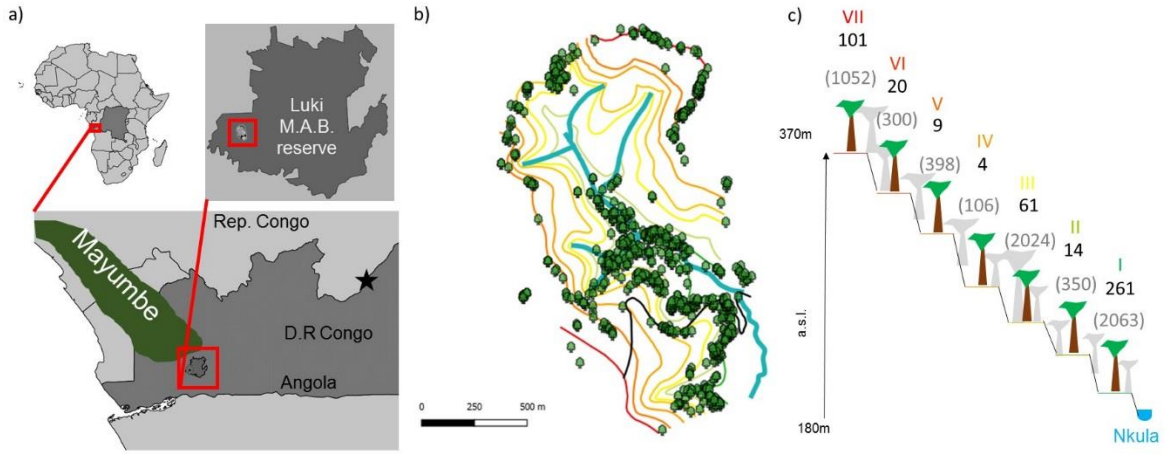


Figure 6.1 Site description (a) The Luki reserve at the edge of the Mayombe; (b) the Nkulapark map, reconstructed from the original map and georeferenced with indication of the rediscovered trees (green dots) from 1947 on (c) transects that follow the relief contours of the catchment; original sample size per relief contour between brackets and retrieved sample size in bold.

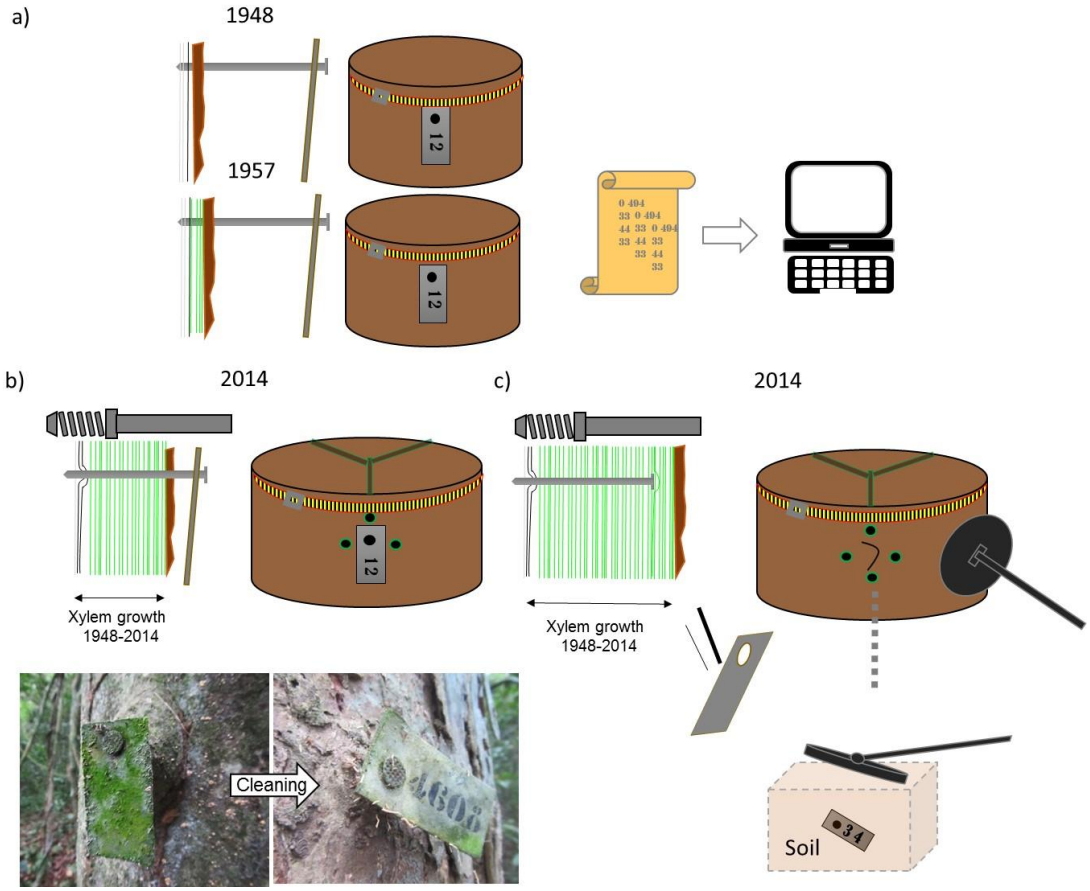


Figure 6.2 Overview of data with (a) Trees of the original study design with yearly diameter measurements until 1957 that were digitized. (b) Trees retrieved in 2014 with original 1948 number tag (b) visible, or (c) overgrown by the tree and the nail was tracked and the tag recovered from the soil, and cores or sections were taken adjacent to the nail to study the cambial mark of the nail, along with additional cores in two directions.

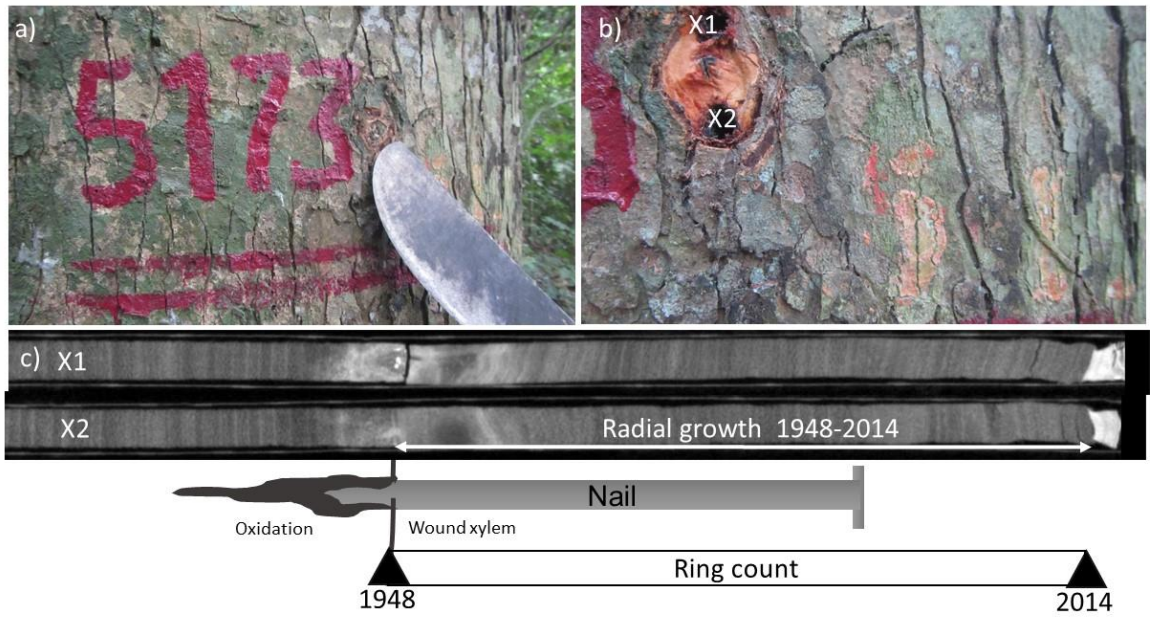


Figure 6.3 Illustration of the sampling and analysis procedure. (a) Prioria balsamifera tree with a mark of the nail that carried the numbered tag completely overgrown by the tree; (b) above and below the nail mark, cores were taken and analyzed using the (c) X-ray CT toolchain: the cambial mark is clearly seen, distances can be measured and rings counted.

6.3 Results

The 10 year (1948-1957) and 57 year (1957-2014) diameter growth is presented in Fig. 6.4.

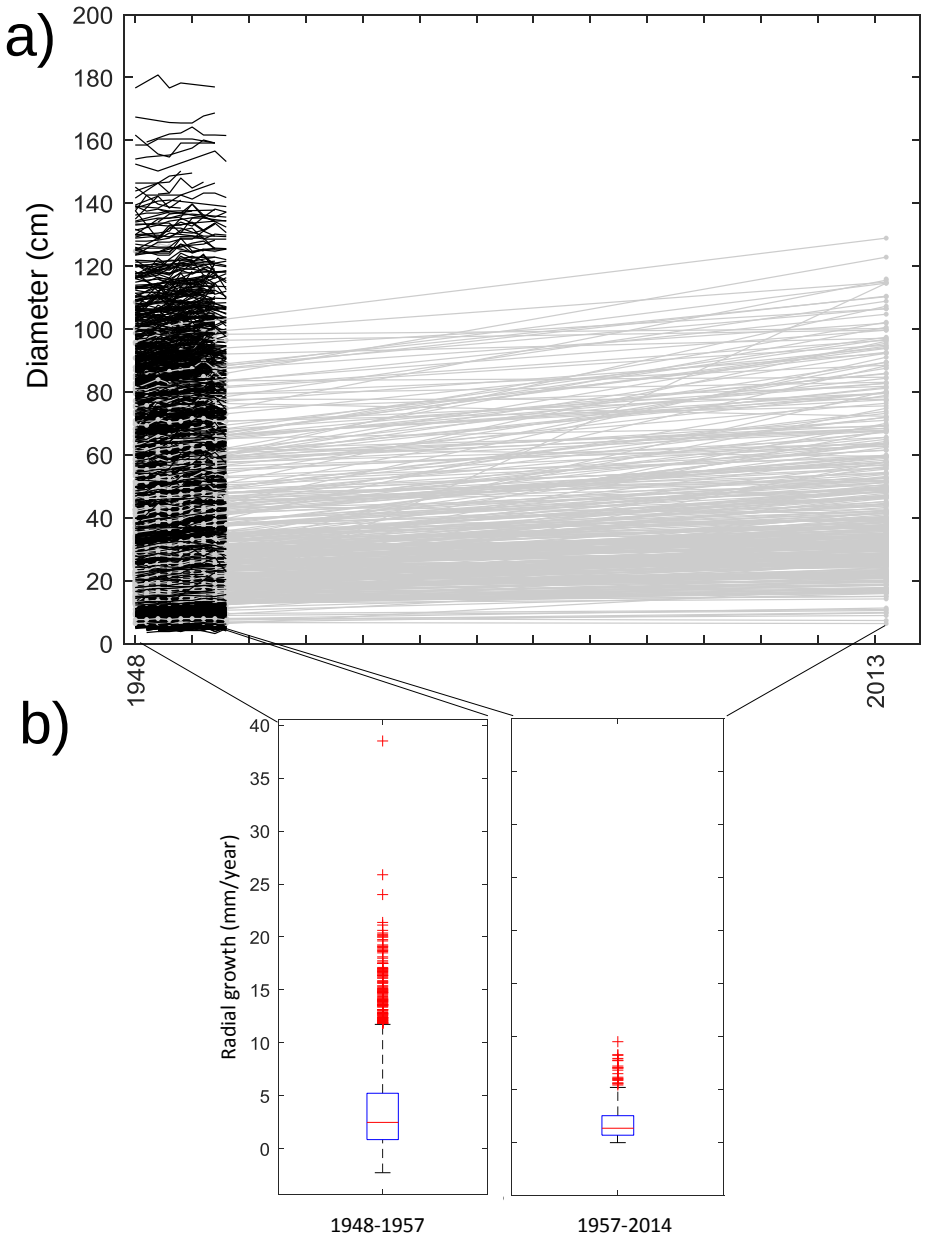


Figure 6.4 Radial growth rates. (a) 10 year archived diameter (black) from 1948 to 1957 and 57 year (grey) diameter growth from 1957 to 2014 (b) boxplot of both periods..

6.3.1 Diameter growth 1948-1957

Median annual radial growth is 2.5 mm (Fig. 6.4) based on measuring DBH in the 1948-1957 period. When looking at the species level, *Terminalia superba*, *Milicia excelsa* and *Piptadeniastrum africanum* show the largest growth. Species such as *Vitex welwitschii*, *Zanthoxylum gilettii*, *Microdesmis puberula* show only a very small diameter growth (Fig. 6.5).

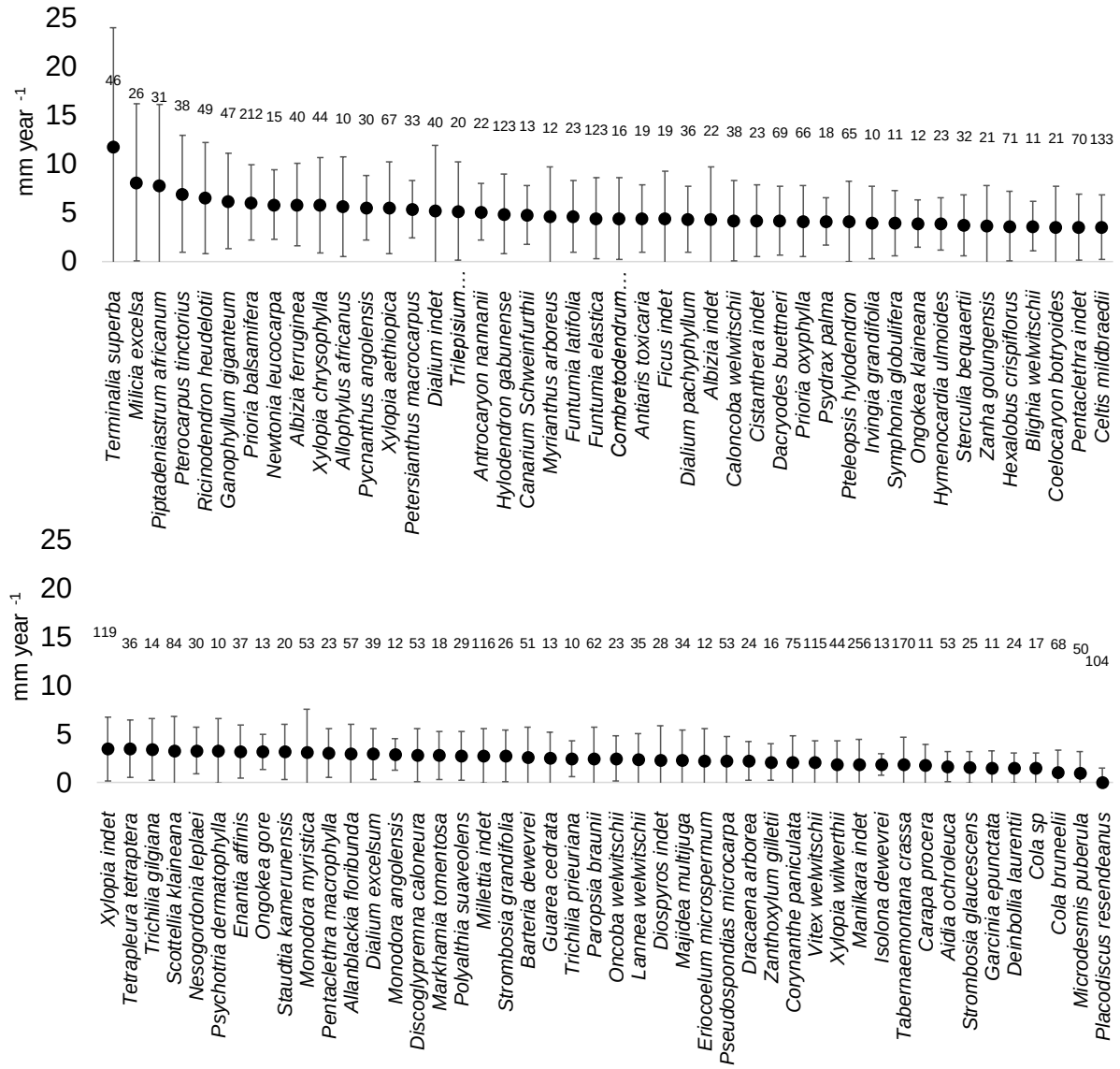


Figure 6.5: Yearly diameter growth from 1948-1957 based on DBH measurements, number of replicates is mentioned above bars. Only species with minimum 10 replicates are shown.

6.3.2 Diameter growth 1957-2014

Median annual diameter growth is 1.36 mm (Fig. 6.4), yet this is highly species-dependent. *P. oxyphylla, P. balsamifera* and *P. macrocarpus* have considerable diameter growth after 66 years (Fig. 6.6), *A. ochroleuca*, *H. gabunense* and *X. wilwerthi* show only little diameter growth whereas *Microdesmis puberula*, *Cola* sp., have no diameter growth.

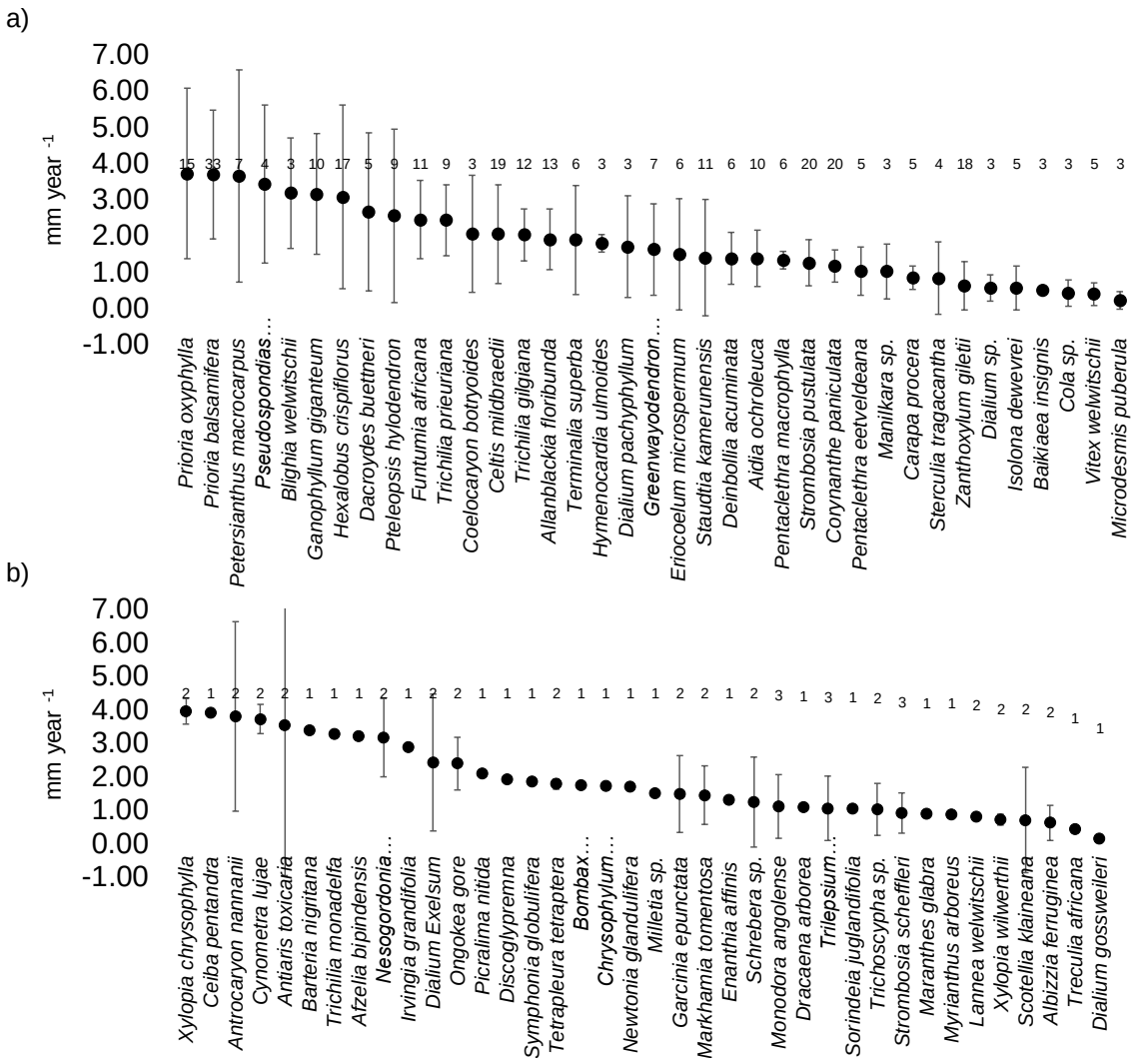


Figure 6.6: 1948-2014 diameter growth for the retrieved species based on DBH measurements, number of replicates is mentioned above bars. (a) all species that have >3 replicates, (b) species with < 3 replicates.

6.3.3 Relation between 1948-1957 and 1957-2014 growth per species

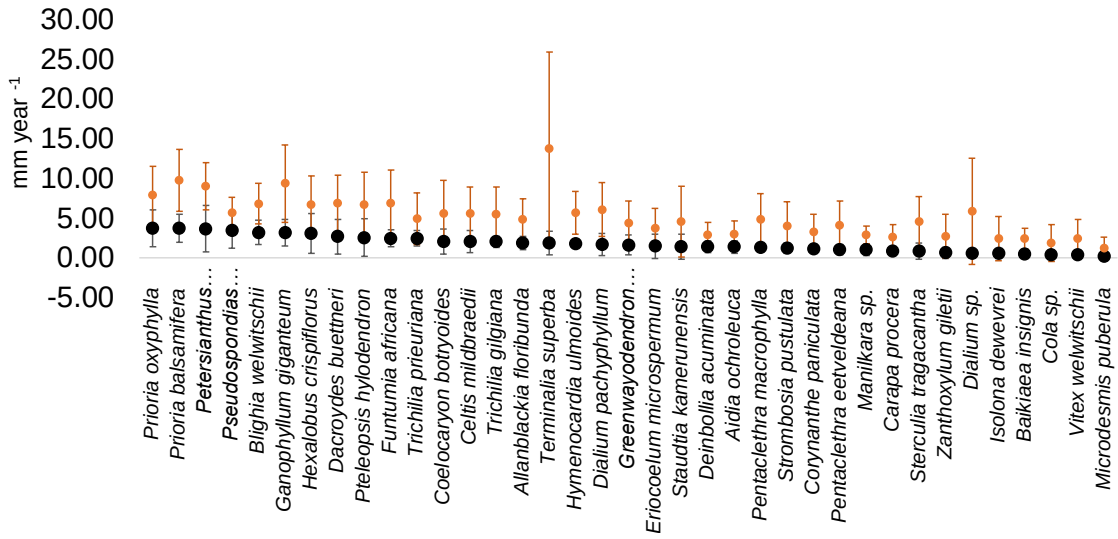


Figure 6.7 Comparison between annual diameter growth per species after 66 years (black dots), and 10 years (orange dots)

Overall, on the same individuals the annual diameter growth from the 57 year period is significantly lower than the 10 year increment (P=0.00, Wilcoxon's rank sum test), and on species level, shifts occur in annual rates (Fig. 6.7). Abundant species in the original dataset, such as *Ricinodendron heudelotti*, *Millicia excelsa*, *Xylopia aethiopica, Albizzia ferruginea* were not retrieved in 2014. Instead, the 2014 dataset shows *P. balsamifera*, *P. macrocarpus*, *F. elastica* and *P. oxyphylla* as the tree species with the largest diameter increment between 1957 and 2014. *T. superba* shows the largest decrease in diameter growth rate.

10 year as well as 57 year radial increment per species is shown in Fig. 6.8 for canopy species, and in Fig. 6.9 for understory species.

Some canopy species show variation in growth rate between individuals based on the 1957-2014 interval. For smaller canopy species and understory species (*G. suaveolens* Fig. 6.8, understory Fig. 6.9), the original diameter distribution is rather small and growth rates are similar both between individuals as in time.

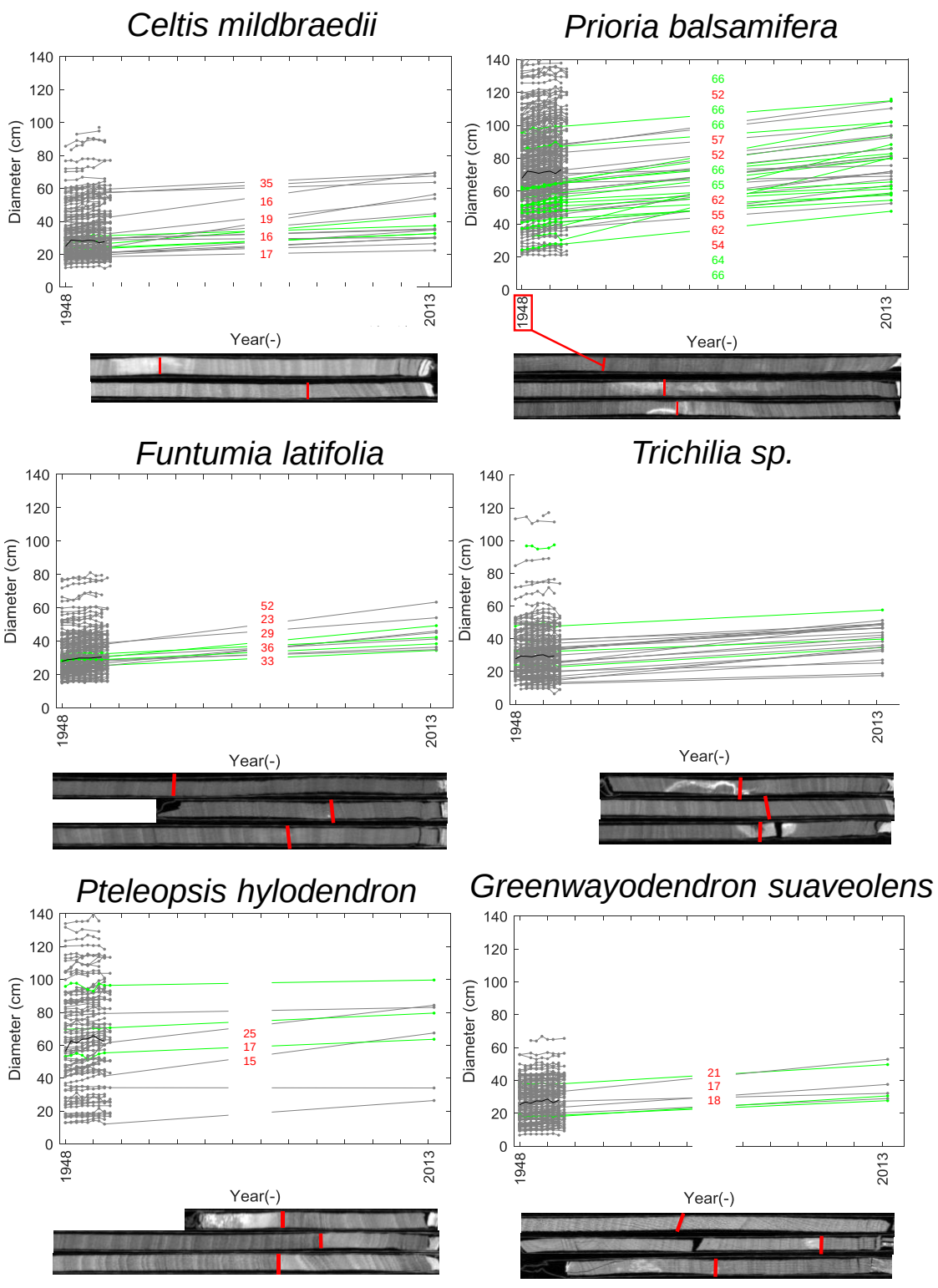


Figure 6.8 Individual 10 year (grey, black=mean) - and 66 year growth (black=retrieved, green= core or sample taken from cambial pinning) for canopy and non-pioneer light demanding species. Xylem formation is illustrated using X-ray CT cross-sections or flatbed images from different trees. Number of discernible rings counting from the visible nail mark of 1948 is given in red, and, when approaching the true calendar years, given in green.

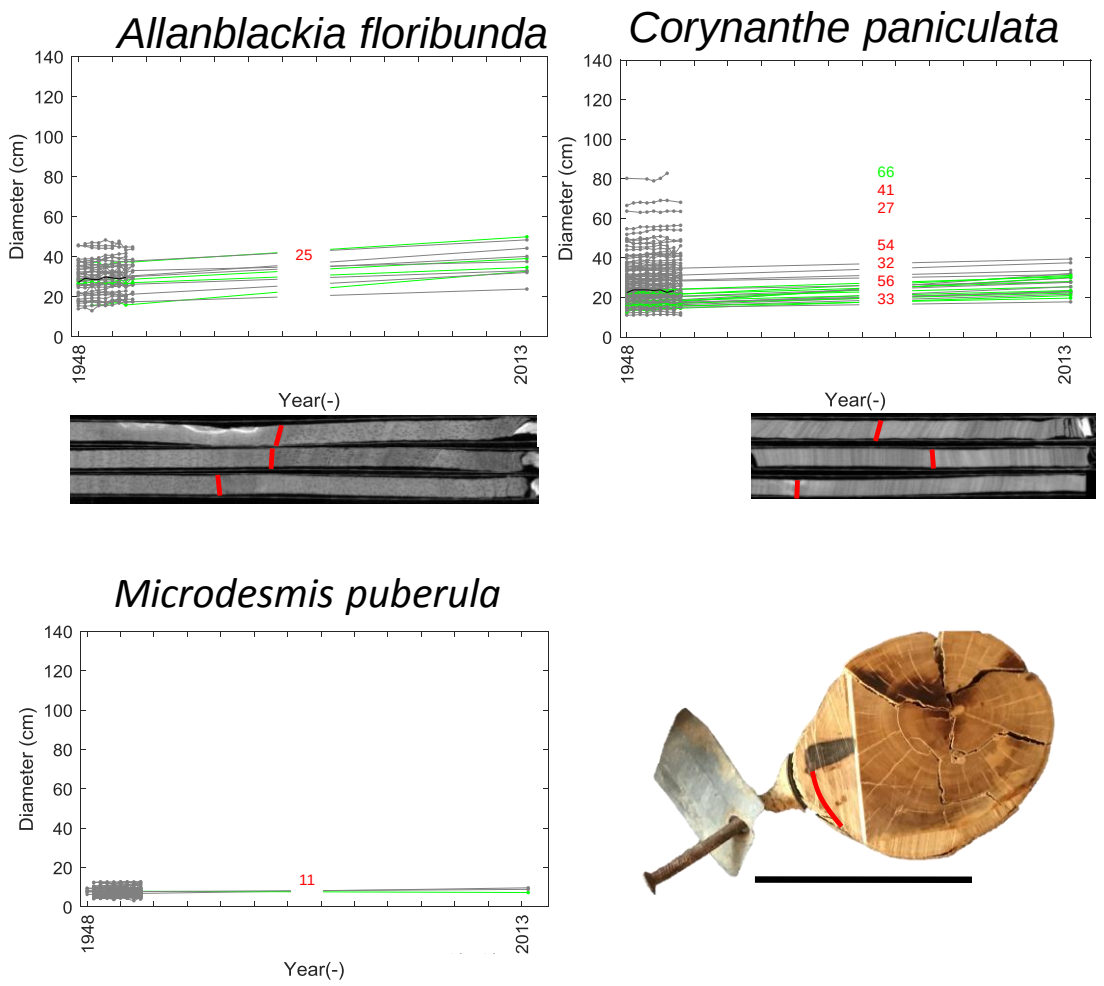


Figure 6.9 Individual 10 year (grey, black=mean) and long-term radial growth (black=retrieved, green=sample) for understory species. Number of visible rings from the mark in 1948 is given in red, and, when approaching the true calendar years, given in green. Scale 8 cm

6.3.4 Nail traces and tree-ring analysis: inter-individual variation

From the set of retrieved trees, samples of fast and slow growing species (Fig. 6.7, 6.8, 6.9 indicated in green) and with little stem irregularities or wound tissue were selected. In general, there is a good agreement between the diameter-based increment and the distance measurements between nail trace and the cambium (Fig. 6.10a).

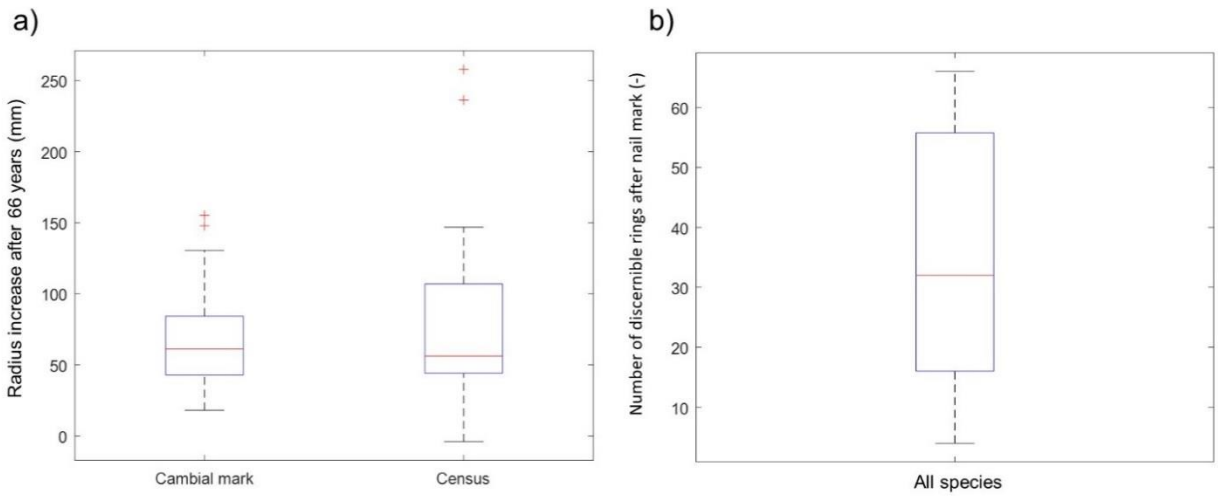


Figure 6.10 (a) Comparison between the distance measurement between the cambial mark and the cambium, and the diameter measurements and (b) number of discernible rings after the nail mark from 1948.

Generally, all sampled trees had discernible rings when visually assessed with the stereomicroscope (Fig. 6.11 a) or when investigating the density profiles superimposed on the X-ray CT images (Fig. 6.11 b). For *P. hylodendron*, *C. paniculata*, *C. mildbraedii*, *G. suaveolens*, a density increase towards the ring boundary is observed, whereas for *T. gilgiana*, *P. balsamifera*, *A. floribunda* and *F. latifolia*, the tree ring boundary starts with thick-walled fibres and ends with fibres with bigger lumina, and vessel size/frequency decreases. The median number of rings is 32, about half of the number of years elapsed (Fig. 6.10 b). The number of rings per species can be found in Table S5.1.

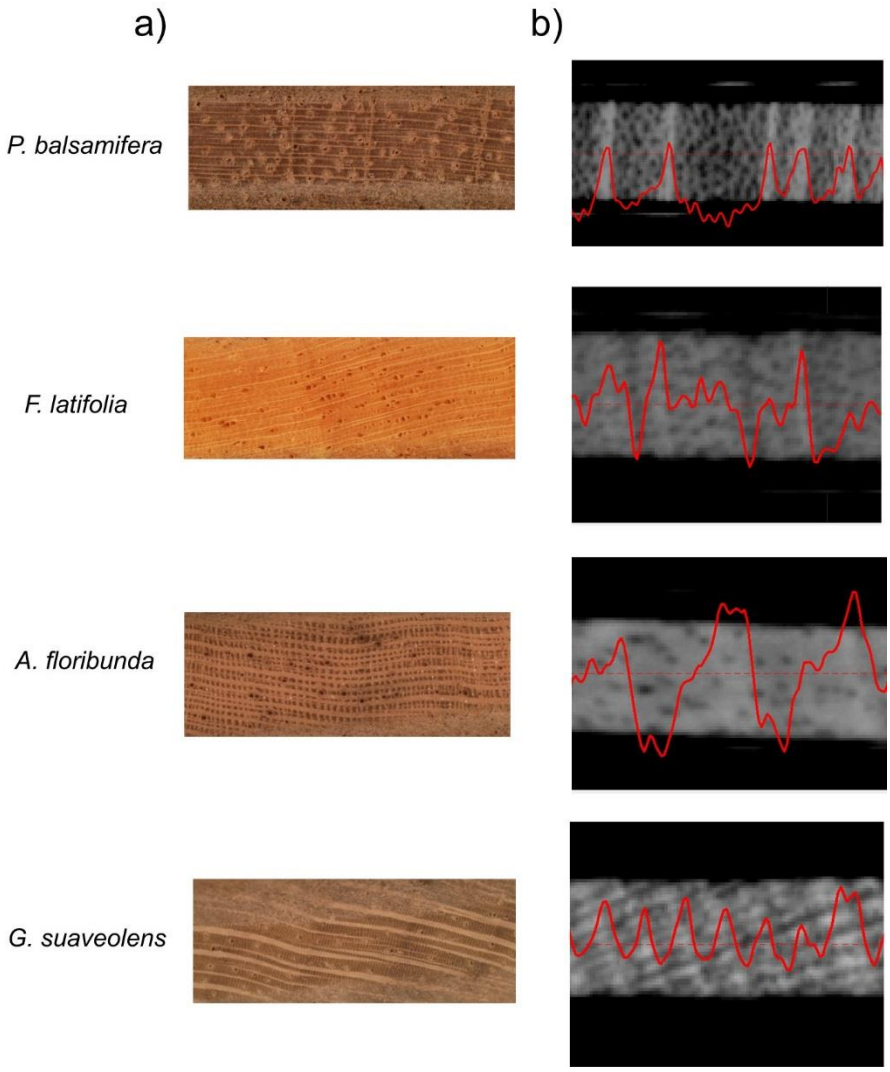


Figure 6.11 Xylem structure of some species, with a) transversal view of the surfaced cores and b) transversal view of the X-ray images with density fluctuations

Both in canopy (Fig. 6.8) as well as understory species (Fig. 6.9) fast growers with wide rings (*P. balsamifera*) and slow growers with only few rings are present. *P. balsamifera* has potential to be used in dendrochronology, given that for 30 % of the individuals approximately 66 years could be counted from the nail trace from 1948 onward (Fig. 6.8). The other individuals had multiple missing rings (Fig. 6.8, *F. latifolia*, *C. mildbraedii*). One individual of *C. paniculata* had 66 tree rings (Fig. 6.9). Remarkably, the sampled individual of *M. puberula* (Fig. 6.10) shows only 11 anatomically discernible rings after 66 years of growth (Fig. 6.12), often only a few fibre cells wide and characterized by a fibre lumina decrease. Furthermore, these rings were counted in the wound-induced local growth (see 5.4.3) zone adjacent to the nail, where rings are wider. Rings are even more difficult to observe further from the wounding. For species such as *F. latifolia* and *P. hylodendron,*

the number of discernible rings is also limited yet these species have considerable radial growth.

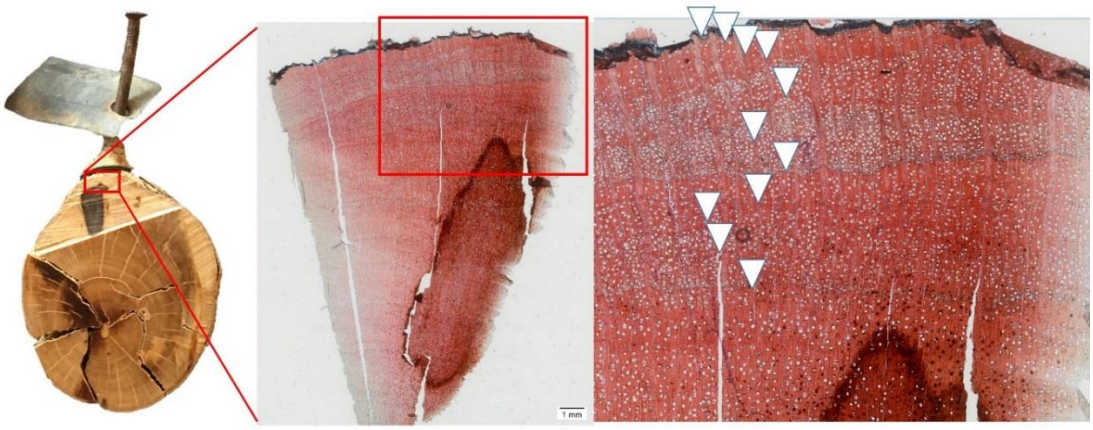


Figure 6.12: (a) Microdesmis puberula showing only 5 mm radial growth and 11 discernible after 66 years

6.4 Discussion

6.4.1 10 years vs. 57 years of radial growth

Both the original and retrieved dataset contained canopy, understory, heliophilous species and pioneer species. Although this classification is not always straightforward due to the large number of species (Swaine *et al.* 1987), it indicates that the species sample set represents all functional groups of the moist tropical forest.

Radial growth from 1948-1957 and 1957-2014 in this study is slightly lower than generally reported in the tropics (Bowman et al., 2013), yet Vieira *et al.* (2005) found similar values based on radiocarbon analysis in the Amazon basin. The discrepancy between 1948-1957 and 1957-2014 measurements for faster growing species is probably due to natural forest dynamics, such as the consequences of mortality of pioneer species such as *R. heudelotii*, of which we retrieved no individuals. Similar evidence is found for the savannah colonizer and pioneers (Hawthorne, 1995) *X. aethiopica*, *H. ulmoides* and *A. ferruginea*, indicating a possible succession from savannah to forest. Illegal logging of certain species is also a possible explanation for the low number of retrieved trees of species such as *M. excelsa* and *P. tinctorius*. Non-pioneer light demanding species such as *P. balsamifera*, *P. macrocarpus*, *F. latifolia* and *P. oxyphylla* appear later in the succession and have a growth maximum at a higher age, possibly explaining the higher growth rate compared to faster

growing species. In addition to the individual age trend (e.g. as observed for *T. superba* where diameter growth has drastically reduced), i.e. all trees that have been rediscovered obviously have aged, the shift from pioneer tree species in 1957 to canopy species in 2014 could explain the smaller increment in the long-term diameter data (Fig. 6.7), compared to the short term diameter differences. Trees classified as pioneers (Hawthorne 1995; Couralet *et al.* 2013) such as *Z. gilettii* and *H. ulmioides* (Fig. 6.7) had a smaller long term radial growth than expected for this guild. Such trees probably remain in the canopy but due to forest closure and thus competition of other trees, they do not grow radially and are merely surviving. The intra-species growth differences reported in Brienen & Zuidema (2007) are also observed in this study for several species, but for *P. balsamifera* little differences are noted. The largest individuals from the 1948 dataset do not appear in 2014, which can be caused by mortality of higher diameter classes, selective logging, or the nail might have been too deep in the wood to be detected, hampering tree identification.

6.4.2 Nail marks as a cambial timestamp

The combination of long term data plots (e.g. Sheil, 1995) and increment core measurements is of particular interest in the tropics (Clark, 2007). By using 66 years old cambial pinnings in this study, the number of (missing) rings can be assessed for a wide range of species and on a longer timespan than commonly performed (Mariaux, 1967; Verheyden *et al.*, 2004; Lisi *et al.,* 2008). Rather than forming small rings, many species tend to form only a few rings of considerable width, or showing little increment without forming distinct rings. The cambial-mark increments of the understory species in this study are also well below the averages mentioned in Worbes *et al.* (2003) based on tree-ring analysis. In general, it is remarkable that many trees show a significant number of missing rings or nonperiodic rings, related to influence of other trees. Moreover, slow growth resulting in missing rings is generally reported in the arctic regions (Wilmking *et al.*, 2012) but is clearly also common in the tropics, more specifically in the understory (Couralet *et al.*, 2010; De Mil *et al.*, 2017). Missing rings have been reported for canopy species as well (*F. latifollia* and *C. mildbraedii* in Fig. 6.8). It should be noted that these might not be missing rings as stated in temperate regions, but rather non-periodical rings. It is important to be aware of this phenomenon: discrepancy between the number of discernible rings at seemingly well-grown dominant tropical trees and age detected via radiocarbon dating has indicated to sum up to multiple decades (Ohashi *et al.*, 2016). Studies relying on absolute dating are thus essential.

Not only is the functional group an explanation for growth differences, also local resource availability (Baker *et al.,* 2003). Assessment of radial growth has to take into account formation of buttresses or lobate growth during the investigated period, which makes diameter measurements problematic (Hawthorne, 1995), and as a consequence, analysis of the xylem structure. For such old trees, increment at lower positions in the stem probably only occurs on the buttresses (cfr. *T. superba, P. africana*) and requires careful interpretation. This also counts for the nail traces, as some nails were present on or between buttresses (Supplementary information Fig. S6.3).

Some trees clearly have an age trend in their ring series, with very small growth for the last decades (*H. gabunense*, *L. welwitschii*), while others showed abrupt growth changes (Worbes, 2004) or had higly suppressed ring growth such as observed by Brienen *et al.* (2016).

Studying understory species thus proves to be highly complementary to the study of well documented and commercial canopy species and faster growing individuals. Whereas tree rings get smaller and ring wedging increases with tree age (Groenendijk *et al.,* 2015), many canopy and understory samples from this study show no age trend whilst the cambial mark indicates that they only formed two or three rings, often of relatively wide ring width. These can be considered non-periodic rings and thus complicate a dendrochronological interpretation (Fig. 6.9, 6.11) and need further confirmation with radiocarbon dating. For the tree to stay alive and to respire, the phloem has to be renewed and assimilates need to migrate from the crown to the roots in order to be functional: the large fraction of missing rings (Fig. 2.10 b), or non-periodical rings is due to the high degree of influence from upper story and neighbours, probably causing biomass increment only in the upper crown (Fritts, 1976; Larsson, 1994; De Mil *et al.,* 2017) or branches.

In many cases, the contribution of wound-induced local growth is considerable, which was also observed in Chapter 4. The question remains whether cambial activity is periodical in trees such as *M. puberula*.

Given the high number of missing rings in trees at their distributional margins, (Wilmking *et al.,* 2012), this assumption could be extended to understory species in resource-limiting environments. This study shows a very slow growth rate of understory species, as such that they could remain for a long time in the “equilibrium phase”, a steady state where trees are not growing (Martinez-Ramoz & Alvarez-Buylla, 1999). Small understory trees with little increment can reach high age (Vieira *et al.*, 2005) and should be further investigated. Furthermore, given their small size, they are less prone to mortality due to drought

(Rowland *et al*., 2015). Consequently, the larger trees are not necessarily the oldest growers (Chambers *et al.,* 1998).

6.5 Conclusion

This is the first study on long term tropical tree growth that combines DBH measurements and xylem growth measurements spanning 66 years. We use the presence of tag nails from 1948 as a cambial mark allowing to obtain multidecadal xylem growth from a historic forest plot.

Differing radial xylem growth patterns across several functional groups ranging from upper story pioneers to understory species were assessed.

Mean annual increment is higher in 1948-1957 than in 1957-2014. A shift in fast growers is seen, mainly from pioneers contributing to the radial growth in 1948-1957, to canopy species in 2014.

The most striking observation is that many species do show a radial increment, but lack a sufficient number of rings (on average 32, instead of 66), thus forming non-periodical rings, indistinct rings or no xylem formation at all. Tree rings of some tree species should thus be interpreted carefully and in combination with other techniques, and might also reveal that understory is much older than can be derived via tree-ring analysis. This study found slow growing trees, unequivocally revealed via the combination of a pre-bomb peak cambial mark and DBH measurements of an old forest monitoring plot. This type of study is rare, but demonstrates that the xylem archive in tropical trees cannot always be assessed via the classic technique of tree ring analysis, but should be investigated in function of wood traits that form these (non-periodical) ring boundaries. They provide the accuracy of measuring carbon sequestration in the trunk, from short timespans (within the season) to multiple decades, and therefore, should be assessed complementary to permanent sample plots.

7

An equatorial view on tree-ring anomalies: opportunities for xylem-trait analysis

Tom De Mil, Jan Van den Bulcke, Joris Van Acker, Hans Beeckman

Preface Chapter 7

Ring phenomena generally described as tree-ring anomalies were detected throughout all chapters. Whereas this was not intended in the original study design, such features are usual in the tropics and deserve a closer look. Chapter 2 reports a missing ring in the growing season 2013-2014 for two of the four T. superba trees. Chapter 4 has demonstrated that small rings carry insufficient information to perform pattern matching based on intra-annual traits. Chapter 5 shows that many samples show only wound-induced growth, illustrated by monthly cambial pinnings in the two monitored seasons. Chapter 6 confirms missing, non-periodic rings and discontinuous rings for many species of the Mayombe.

Aforementioned phenomena are therefore often perceived as problematic in tree-ring research. However, these "problems" are rather rule than exception in the tropics, and can also be recognised as potential opportunities, in some cases even in terms of intra-annual wood traits, which is discussed here.

7.1 Introduction

Knowledge on tropical tree age and growth under a changing climate is vital to predict future carbon emissions and the response of tropical forests. Permanent sample plots in tropical regions are increasingly being established for monitoring forest growth, but the oldest plots are not ranging back more than a few decades. To assess long-term trends in tree growth, the xylem is explored via tree ring analysis. Methods in routine tree ring analysis are traditionally designed for higher latitudes but have been successful for many tropical species in different regions along the tropics. In boreal regions, dendrochronology based on ring width or other single-value parameters such as Maximum Latewood Density (MXD) seems sufficient and has contributed to climate reconstructions ranging back thousands of years. For more mesic sites in temperate regions, tree ring dating is evident, but research is conducted to add intra-annual information to these ring widths, investigating the plastic response of the xylem tissue to elucidate its climate sensitivity (Fonti *et al.*, 2010; von Arx *et al.*, 2014). In tropical regions, serious effort has been done on identifying tree ring boundaries, based on wood anatomy (Groenendijk *et al.*, 2014; Tarelkin *et al*., 2016). However, undiscernible rings, circumferential variation, intra-annual growth variations, wedging and missing/non-periodical rings are commonly described as problems in tree ring analysis, and identified by use of stem disks, often resulting in discarding samples with such problems for further analysis. Therefore, only a selection of key species is considered suitable for tree ring analysis, but this (i) does not take into account the within-ring features and (ii) does not answer ecological questions why some trees show indiscernible rings, missing rings and non-periodic and false rings.

Following the scheme of Wils *et al.* (2010) (Fig 7.1), plotting missing rings and “multiple rings” (intra-annual density fluctuations (IADF)) in function of rainfall regime, rings range from being indiscernible to completely missing. For tropical juniper trees (Sass-Klaassen *et al.*, 2008) it is observed that false rings (IADF’s) occur in years with a bimodal rainfall distribution. For angiosperm trees, intra-annual features have been related to droughts within the rainy season (Mariaux, 1969).

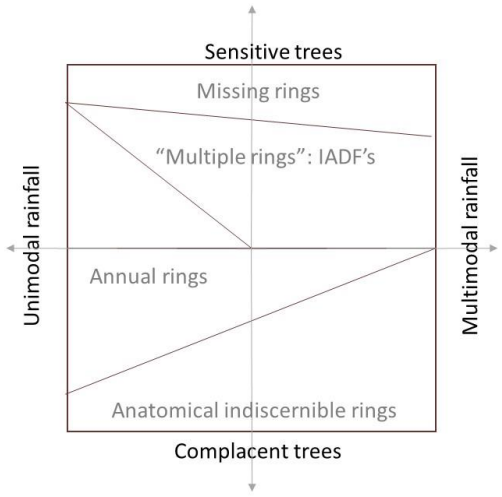


Figure 7.1 Scheme of ring anomalies adopted from Wils et al. (2010)

Often observed in trees at their species' distribution limit in strongly seasonal environments (Wilmking *et al.*, 2012), missing and non-periodic rings also occur frequently in the tropics (Ohashi *et al.*, 2016) and it is more difficult to detect them via cross-dating, as no extensive chronologies are available.

The above framework, is a simplified diagram based on trees with a theoretical radial geometry, not taken into account internal variation of the stem, around the circumference. Geometry can be complex, and has not been studied physiologically, while it is shown to have many variations exist, especially in the tropics (Lachenbruch & McCulloh, 2014).

One of these variations is wedging rings, which are not described in Fig. 7.1, and are defined as partially missing rings frequently encountered in tropical species (Worbes, 2002) and could occur due to lack of resources at a certain stem position (Speer, 2010). They are considered as a phenomenon causing problems in cross-dating (Chapter 4, Groenendijk et al., 2014), but are known to occur in years of extreme droughts (Sass-Klaassen *et al.*, 2008), and can thus be an indicator of tree response to climate.

As such, ring phenomena in tropical trees range from indiscernible to (partially) missing rings and non-periodical rings, whether or not with deviations in geometry of the cross-section.

This chapter summarizes the phenomena that were encountered when monitoring and analyzing trees during this work, assessing them as potential opportunities rather than anomalies.

7.2 From indiscernible to IADF, wedging and eventually missing rings

The majority of the samples from this work have discernible growth rings, seen macroscopically, via microsections, or via X-ray CT imaging (Chapter 6).

7.2.1 Intra-annual variations and circumferential variability

When checking on stem disks in *T. superba* trees from chapter 2, intra-annual density fluctuations were present, and on the widest rings they were more discernible. Therefore, in accordance with Eglin *et al.* (2010), the circumferential section containing the widest ring (T4) has been sampled to maximize intra-annual information.

When comparing two rings of the same year of tree T4 (Table 2.1) in *T. superba* (Fig. 7.2), it is clear that the widest rings provide a higher spatial, and thus, a higher temporal resolution for assessing traits. Sampling a wider ring increases the temporally resolvable limit, also given the minimum amount of wood necessary for isotope analysis (Poussart *et al.*, 2004) and for quantitative wood anatomy measurements. Two clusters in vessel distribution can be found (Fig. 7.2a). As IADF's are only seen in wider rings (Abrantes *et al.*, 2013), this would justify sampling the widest rings in *T. superba* to shed light on the plastic response of the xylem. Despite improvements in measurements, such as for isotopes (Schollaen *et al*., 2014), wood anatomy (von Arx *et al*., 2016), there has to be a sufficient amount of sample depth for intra-annual analysis of traits. Wood anatomical traits such as vessel size and frequency, are biologically restricted to a minimum cell size as the smallest measurable unit (e.g. vessel area), so that widest rings contain the most information.

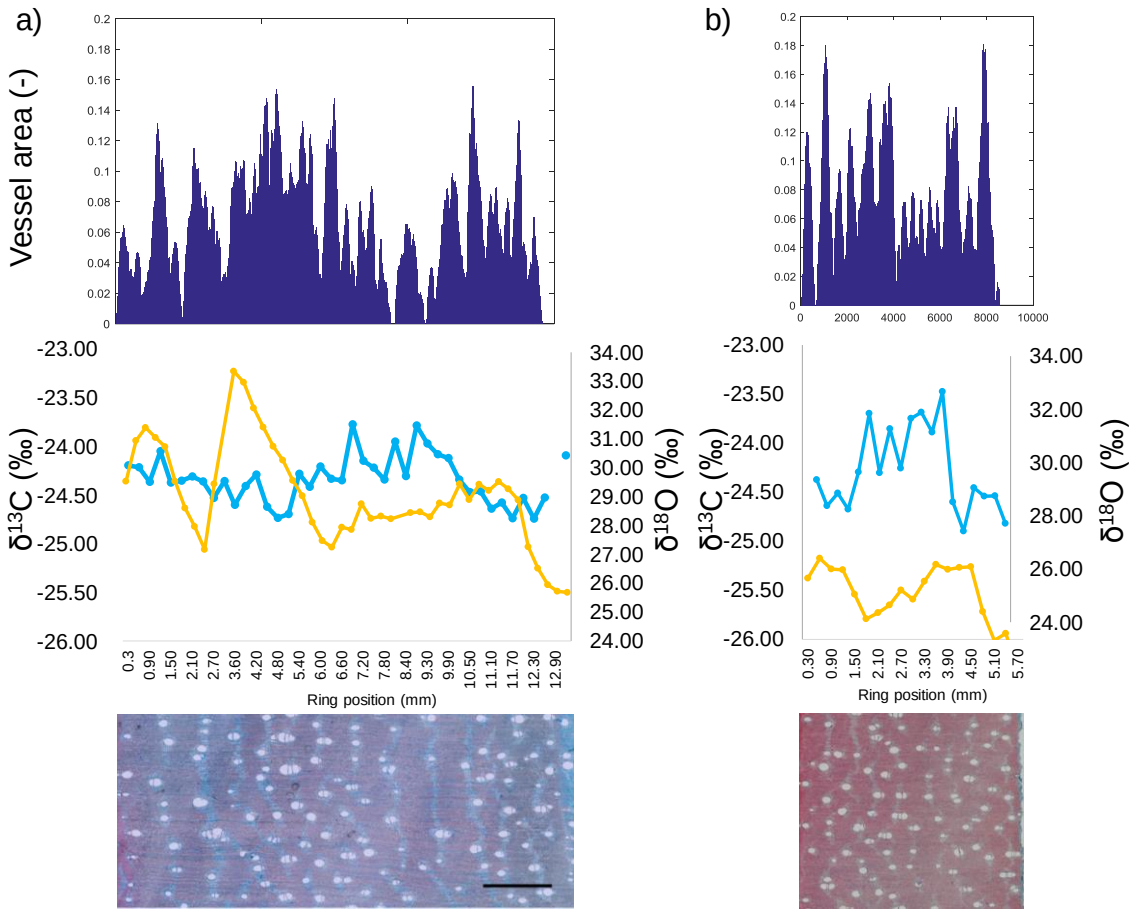


Figure 7.2: Illustration of a (a) wide ring and (b) small ring both formed in 2014-2015 on the same tree, with wood cellulose $\delta^{13}C$ (yellow) and $\delta^{18}O$ (blue), and relative vessel area.

7.2.2 Wedging and missing rings

<u>Wedging</u>

Wedging is described in Chapter 5, and can be considered as an extremer case of circumferential variability, where the cambium does not produce xylem all around the circumference due to geometry.

The same assumption as in selecting the widest rings (7.2.1) holds when wedging occurs: selecting the widest section for intra-annual trait measurements. Just as postulated by Worbes (2002) in searching the widest rings for tree ring analysis, for intra-annual xylem traits research the same methodology of “broken radius” could be applied, i.e. selecting the widest rings from pith to bark by moving along the circumference, rather than drawing a straight line.

Missing rings

Radius dendrometers were used to assess Stem Diameter Variations on 4 *T. superba* trees, where two trees showed missing rings, without any exceptional drought.

In Chapter 5, missing rings were spotted on 34 % of the trees with the technique of monthly cambial pinnings. In Chapter 6, a 66 year pinning experiments showed on average 32 rings on all sections. For intra-annual trait research, little can be assessed in retrospect as no xylem is formed. Figure 7.3 shows stem diameter variations of a *T. superba* tree with and without a missing ring. The dendrometer radius increases after the first rains, then reaches a plateau, with interruptions due to intra-annual droughts, followed by a return to its original position, without any net growth. This study is the first to report a missing ring in “real-time” based on SDV series and was further verified via cambial pinning. SDV series can be considered a proxy for TWS when radial growth is eliminated from the measured signal (Zweifel *et al.*, 2001; Steppe *et al.* 2006), especially for species with a relatively thick bark (Borchert, 1994). In this case, xylem growth is non-existent; the curve thus only represents water status, which is an opportunity for deriving tree water deficit from SDV series.

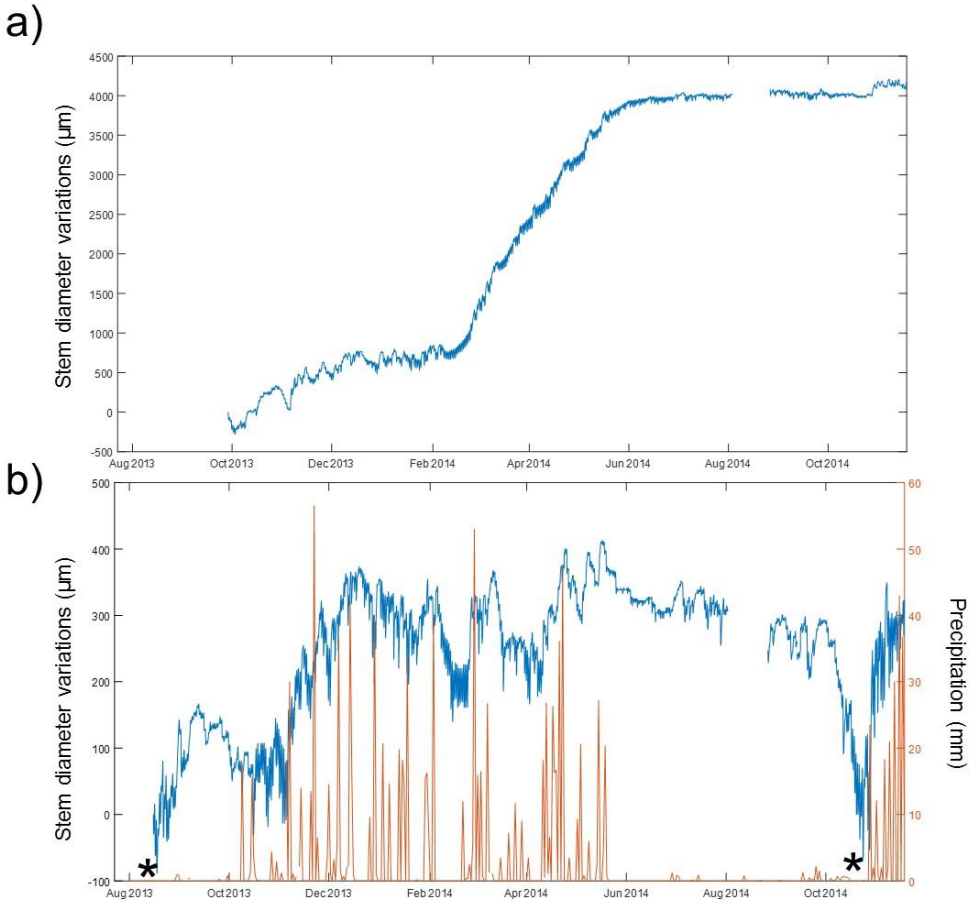


*Figure 7.3 Live monitoring during the 2013-2014 season shows a SDV series of a (a) tree with xylem growth and (b) a tree that did not form a ring, where the radius returned to original position (marked by *). Precipitation data is plotted as well (orange).*

DBPM (Chapter 4), takes into account at least some wedging rings and missing rings due to its shifting procedure. However, when too many rings are missing as observed in the study on the understory species, combined with generally small ring widths with insufficient density information, missing rings cannot be detected.

In chapter 5, missing rings were spotted on 34 % of the trees with the technique of monthly cambial pinnings. In chapter 6, a 66 year pinning experiment showed only 32 rings on average.

Given the large occurences of missing or non-periodical rings in all species from this study, they cannot simply be discarded for further analysis. Non-growing trees are an inherent part of the tropical forest, and thus should be further investigated. Ecophysiological measurements of these trees can reveal climate response, without being seen in the xylem structure.

Next to serial sectioning (Wilmking *et al.*, 2012), future work should be focussed to monitor leaf/flowering phenology and perform cambial pinnings and dendrometers at multiple positions on the circumference (Fig 7.5b) as in Chapter 2 performed for *T. superba*. Moreover, multiple positions along the length axis of the trunk (Fig 7.5a) should be sampled and monitored.

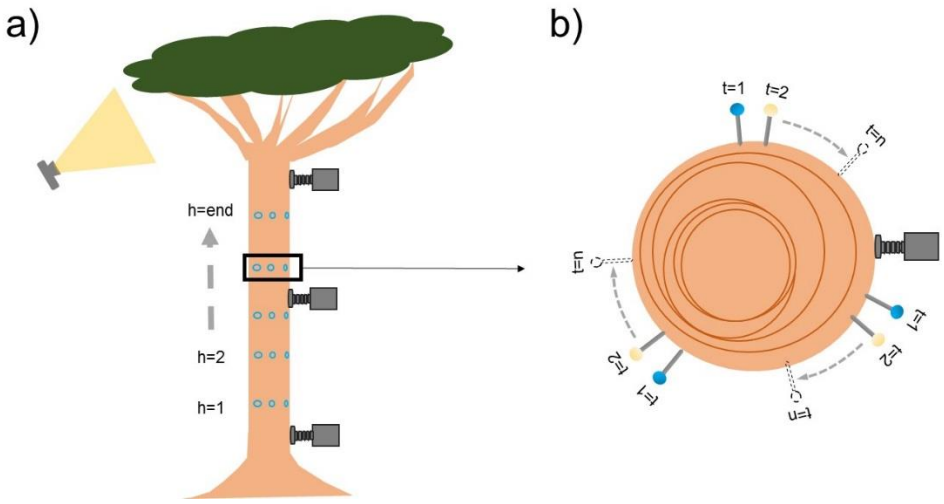


Figure 7.5 Possible sampling scheme to investigate wedging, missing or non- periodical rings. Leaf and flower phenology monitoring combined with cambial phenology a) along the length axis of the trunc and b) along the circumference.

For all monitored trees that showed no xylem growth, we did observe wound-induced growth, characterized by a different wood anatomy compared to normal growth (Arbellay *et al.*, 2012). It is therefore advised to be careful when sampling close to the pinning, as "false" radial growth is measured.

Especially for understory species, their biomass investment could be mainly absorbed on wound formation, as is shown in the samples that show only wound-induced formation after 66 years (Fig. 7.6).

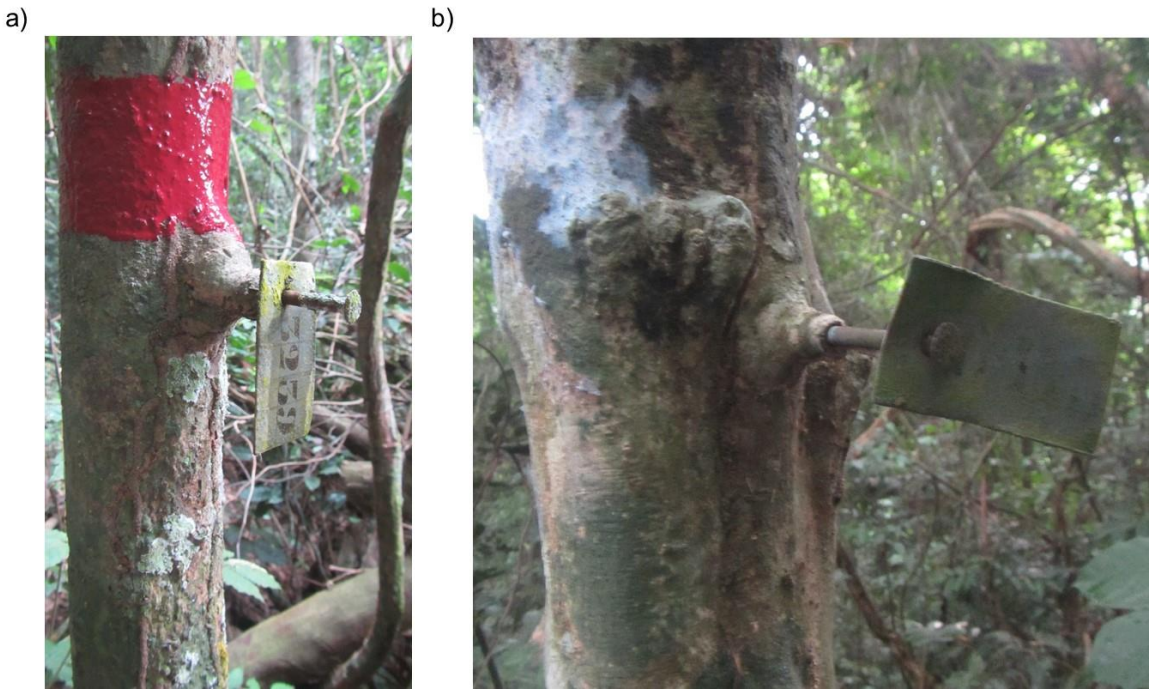


Figure 7.6 Understory trees measured in 2014, are showing no radial growth since 1948, except for wound formation due to the nail. a) Cola sp., b) unidentified species

Eventually, radiocarbon dating on some trees should give an idea on the number of missing rings, but is problematic when the tree age situates after the 17th century or before 1960 (Chapter 6).

7.3 Cambial phenology as a driver for ring variations

The samples from the Mayombe site in this study illustrate that intra-annual density fluctuations and missing rings appear to a varying extent across species in the same forest, between trees, and eventually within the tree trunk. The scheme in Fig. 7.1 should thus be expanded to a wider context, where the intrinsic factors such as interference of neighbouring trees will also have an effect on the wood structure: (i) not only between functional groups differences occur (Chapter 5, 6), also (ii) between trees of the same species that differ in social position, size (Rathgeber *et al.* 2011), age. (Chapter 2). Both inter-species as intra-species variations in wood structure are caused by leaf and cambial phenology differences (Rossi *et al.*, 2008).

In general, a possible explanation for the occurrence of both missing rings and intra-annual density fluctuations (Fig. 7.7a) in the same forest, whether or not with geometry variations

(fig. 7.7b) could be found in the cambial phenology (Fig. 7.7c). In the first case, the tree is suppressed (Chapter 5, or T1 in Chapter 2), small rings are formed, and the phenology timing would shift to a later onset and/or earlier cessation (Rathgeber *et al.,* 2011). In extreme cases, this can lead to non-periodical rings causing only little radial growth after multiple decades (Chapter 6). Detailed crown descriptions and indices such as the crowding index (Michelot *et al.,* 2011) should be used in order to take into account site and competition influences. In the second case, a small stagnation occurs in the growth curve, forming an intra-annual trait variation during that year (e.g. T4 in Chapter 2), as is illustrated in Steppe *et al.* (2015). Moreover, the bimodal flowering pattern (Chapter 5) as shown in Couralet *et al.* (2013) could imply that species are adapted at growing during a certain period within the season, and functional groups can be an explanation for differences in cambial phenology (Couralet *et al*., 2010). In this study, strong intra-tree variation in leaf phenology between branches of the same tree (Klebs, 1912, Alvim 1964 in Worbes, 2002) was not observed, thus probably did not affect circumferential variation.

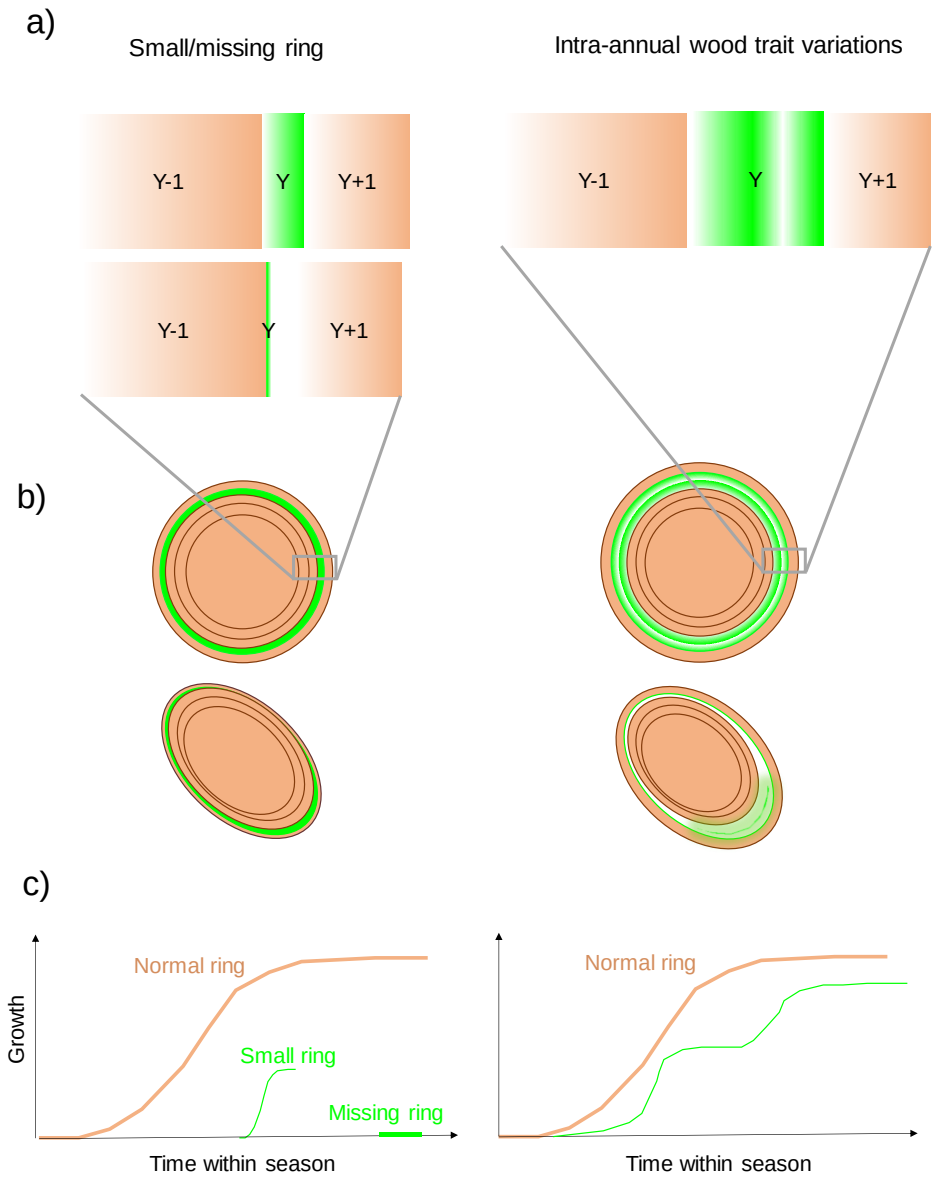


Figure 7.7 Illustration of ring width and its possible relation with cambial dynamics. a) illustration of both small and missing rings, versus intra-annual trait variations b) Radial geometry versus other geometries, c) possible cambial dynamics associated with these ring phenomena.

7.4 Conclusion

Trees from this study site in the Mayombe show circumferential variation, wedging and missing/non-periodical rings, thus covering a large part of the spectrum of ring phenomena, in one study site.

Circumferential variation and wedging

Different tree-ring features should be used for a more complete view on the climate-growth relationship, especially in mesic sites (Martin-Benito *et al.,* 2012), and the often moist conditions can cause wide tree rings, that, at first glance, show complacency, but intra-annual traits should be able to give a more detailed view on seasonal response to weather.

The typical circumferential ring phenomena described in this chapter are considered anomalies in traditional tree ring research, but are an opportunity for assessing intra-annual wood traits. This is a similar opportunity as with IADF's in trees from Mediterranean areas, which were treated as an anomaly in the beginning but are increasingly being used as an opportunity to assess climate sensitivity (De Micco *et al.*, 2016). Widest rings have more chance to contain IADF's, which should thus motivate to sample the widest rings within a tree, when geometry variations cause circumferential variability or wedging. Moreover, going further south to the equator, intra-annual traits, whether or not causing a density fluctuation, are perhaps more relevant due to problematic tree ring width based matching.

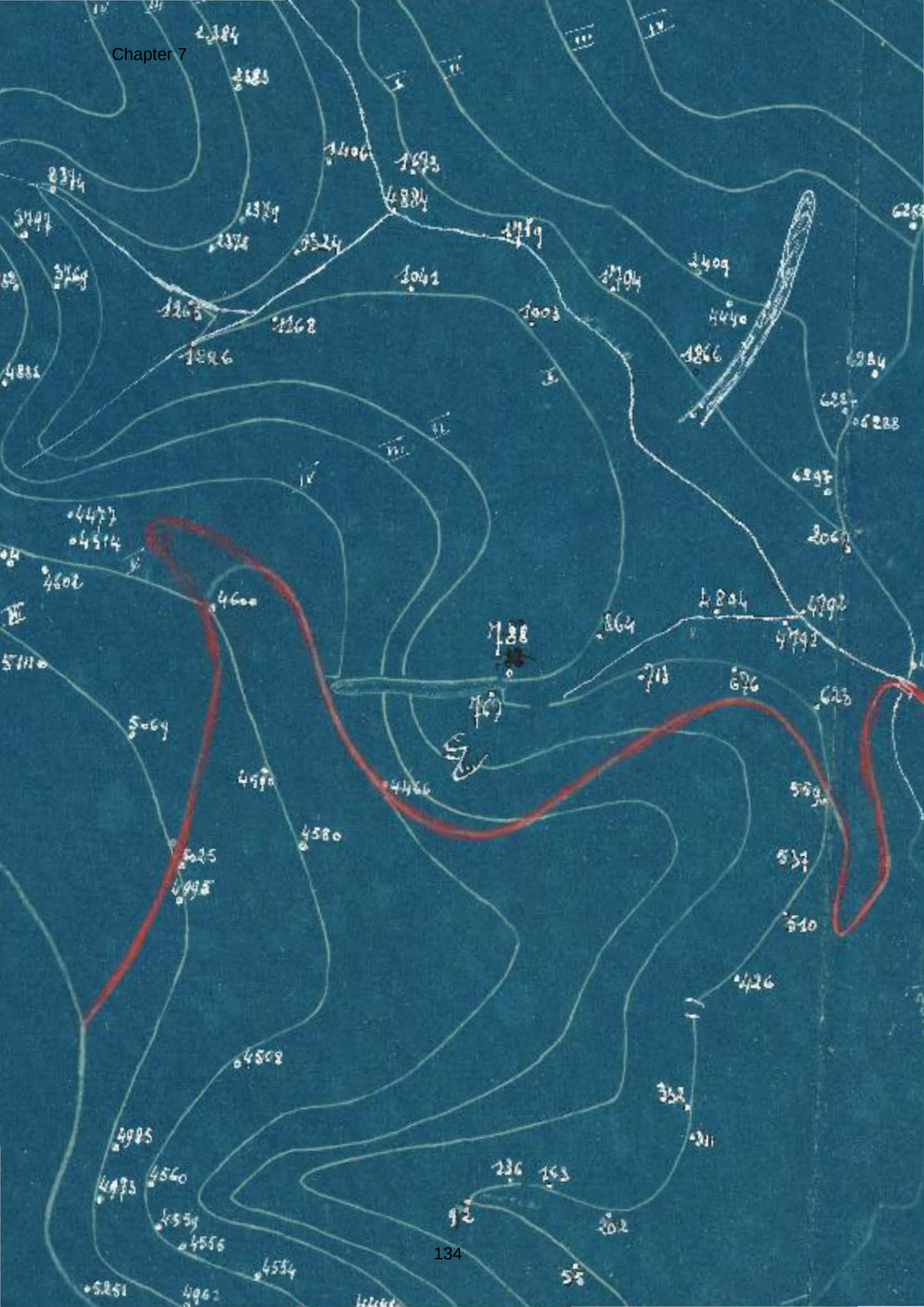
Missing rings

Ring-width based pointer years typically assessed in routine dendrochronology are small rings even down to microrings, measuring not more than a few cells and are indicative of years where the resources for tree growth were limited. Non-periodic and missing rings occurred throughout the chapters in this thesis might also be an indication of response to the environment. Missing rings and small rings could be seen as opportunities as well, but not for intra-annual analysis, rather because their presence could be considered characteristic for certain "events" that hampered biomass accretion. Trees with repeated missing rings should thus be monitored in real-time, as no extensive chronologies exist such as in temperate regions. Not only the pinning technique should be further applied, but also phenology monitoring and ecophysiological methods for deriving water status is necessary, such as dendrometers, at multiple positions and heights.

Species with ring anomalies are discarded due to their non-existence of annual rings, but this does not imply that they are not climate sensitive. Given the fact that under increasing drought, the understory disappears in favour of deciduous species in the tropics (Fauset *et al*., 2012), it is of special interest to perform research on these understory species and climate impact upon them, and why they can remain in a multidecadal steady state phase.

Chapter 6 has shown little xylem growth after 66 years, which was an unprecedented opportunity to challenge the general prejudice about age and growth rate of tropical trees.

A new framework should thus be considered, with long term monitoring of species or trees showing these ring phenomena. To achieve a deeper knowledge on these canopy and understory trees, a list should be made with species that show frequent missing/non-periodical rings or other ring phenomena. Often these species are found on a “blocklist” for tree ring potential in traditional dendrochronology, but might be more prone to climate change than we would expect, except it is not seen directly in their growth ring structure.



8

General discussion and conclusion

Reliable information on tropical tree response to a changing environment is vital for assessing the future state of tropical forests and their role in, amongst others, carbon sequestration. Given that past tree response is imprinted in the wood, there is a need for methodological improvements that reach beyond routine dendrochronology in order to unlock the information hidden in a tree ring.

The rationale of this dissertation is to unravel radial tree growth in tropical trees in the Mayombe, DR Congo. Starting at the intra-annual time scale where we study stable isotope and wood anatomical traits of *Terminalia superba* in relation to cambial and leaf phenology, the inter-annual and multi-decadal scale are assessed through upscaling using X-ray CT densitometry. Finally, cambial phenology was monitored for multiple species, and the X-ray CT toolchain was applied on trees from a long-term forest monitoring plot.

Following objectives were achieved:

(i) Fix xylem traits on a time axis via phenology of apical (leaf) and lateral (vascular cambium) meristems

In Chapter 2 we combined high resolution dendrometer and stable isotope data with wood anatomical traits on mature forest trees of *T. superba* from different sites during 2 seasons, to add intra-annual and seasonal dated information to the challenging analysis of tropical tree response. This approach allowed us to convert xylem traits on an intra-annual distance axis to a time axis.

Variation in leaf phenology timing between trees is observed, but the timing is consistent for each of the growing seasons, despite timing in rainfall onset and SDV-derived stem water content. Leaf abscission continues until late in the dry season, showing the brevi-deciduous character of *T. superba*. In general, cambial phenology follows a similar consistent behaviour, yet lagging up to two months probably due to tree height.

We observe that leaves start developing irrespective of the start of the rainy season for *T. superba*, which is important concerning tree vulnerability to climate change. When trees flush, transpiration starts, irrespective of the weather conditions. Further absence of rainfall at the beginning of the rainy season could lead to severe stresses, followed by decreased growth and eventually tree decline. It also explains the high positive correlation between tree-ring width and November precipitation at the beginning of the season in the Mayombe (De Ridder *et al.,* 2013). The aspect of phenology is thus essential for interpreting intra-annual wood traits to actual climatic or other events during the growing season. Missing rings were observed for two trees in the 2013-2014 season.

Contrasting trends for $\delta^{13}C$ profiles are noted when positioned on a distance axis, as is commonly done in literature (Fichtler *et al.,* 2011), yet when accurately positioned on a time axis by means of cambial phenology, the profiles seem to synchronize. Fixing wood traits to a time axis was necessary for comparing the $\delta^{13}C$ profile between trees. Proper interpretation of the $\delta^{18}O$ signal was less clear, probably due to variations in source water as well as further fractionations.

Compared to stable isotope traits, the wood anatomical traits such as vessel, parenchyma and fibre profile are more likely to be affected by the environmental conditions during that year, because they are directly linked to cambial phenology (Fonti *et al.,* 2010).

Recommendations

It is generally known that intra-annual stable isotope patterns are largely influenced by the previous year(s), thus the associated migration of non-structural carbohydrates needs to be taken into account (Hartmann & Trumbore, 2016) while interpreting the wood cellulose isotope pattern.

We further encourage *in situ* manipulative experiments to unravel the variation in intra-annual wood anatomical traits (Cavaleri *et al.,* 2015; Zuidema *et al.,* 2013) as a response to drought. Further research could focus on detecting tree rings in the past to see how mid-season dry spells affect intra-annual anatomical and isotope series.

Rather than cambial pinning, a more detailed assessment of cambial phenology can be obtained with periodical microcoring, to identify detailed cell formation processes such as number of cambial cells, number of enlarging cells, lignified cells and matured cells (Cuny *et al.*, 2015; Güney *et al.*, 2016). While this is easier to accomplish for conifers, the anatomy of tropical angiosperms with traits varying in size and frequency, complicates such analyses. Therefore, cell formation processes need to be separated for each wood

anatomical trait (such as e.g. vessels vs. fibres and parenchyma), and then linked directly with leaf phenology (Pérez-De-Lis *et al.*, 2016; Kitin *et al.*, 2016).

Fixing intra-annual wood traits on a time axis via cambial phenology causes multiple trees of one species to be synchronized at the intra-annual level, which could enable to estimate cambial and leaf phenology in the past, based on xylem traits.

(ii) Explore X-ray densitometry as a suitable method to describe intra-annual wood traits

Wood density is determined by chemical and wood anatomical traits of the xylem. Due to the tediousness of measuring wood anatomical traits such as vessels, parenchyma and fibres, X-ray densitometry is proposed as an alternative technique since it is fast and non-destructive. In Chapter 3, densitometry was related to intra-annual variation of vessels, parenchyma and fibres in *T. superba*. As a reference, manually measured samples from the last two rings of the trees from Chapter 2 were subjected to X-ray CT densitometry at 35 µm resolution and a faster screening resolution of 110 µm. It was shown that densitometry is able to detect intra-annual features and can give an estimation of percentage of variation of intra-annual variability of vessel area, parenchyma area and fibre variation via principal component analysis. For *T. superba*, the fibre wall fraction determines the largest positive variation in the intra-annual profile. While it is shown that the tree-ring boundary is generally known to be a density fluctuation (De Ridder *et al.,* 2013), the intra-annual signals due to drought will also likely be reflected in the fibres. Vessel area is negatively related to the intra-annual density profile, whereas parenchyma is highly related to vessel area. Mainly the negative relation with vessels and parenchyma, and the positive relation with fibres, suggest that densitometry is a suitable proxy for intra-annual wood traits for *T. superba*.

Moreover, this study demonstrates that rings can be delineated and intra-annual fluctuations in *T. superba* can be detected with X-ray CT densitometry at 110 µm resolution. This allows scanning of even more cores at once compared to scanning at 35 µm, therefore enabling true high-throughput analyses.

Recommendations

The presence of parenchyma and vessels results in high frequent changes hampering tree-ring analysis (Mariaux 1967, Vetter 1995) and is partly observed in this study for *T. superba*, but the 3D volumes can be analysed using advanced image processing routines to tackle this issue. Therefore, densitometry profiles should be decomposed for other species with other tree boundary types as well, in order to assess the applicability of tree ring boundary demarcation based on the density profile.

Not all species will have anatomical features that allow the tree-ring boundary to be delineated based on X-ray CT densitometry, but other analysis techniques such as spectral analysis (Verheyden *et al.* 2005) could disentangle sub-annual signals from annual signals in the density profile. Additionally, more advanced image processing techniques should be tested as well. Furthermore, as criteria on ring presence or absence should be reconsidered (Tarelkin *et al.,* 2016), perhaps we should just focus on intra-annual wood traits, rather than actually defining the boundaries.

Density-Based Pattern Matching, discussed in the next section, is a first step in this direction:

(iii) Develop a toolchain to treat large sets of tropical tree increment cores in a fast and efficient way, and to present a method to enhance tree-ring dating via these intra-annual wood traits

A field-to-desktop toolchain for X-ray CT densitometry was developed in order to upscale the conventional and tedious technique of densitometry for batch processing and data mining of large datasets. Moreover, taken into account the (i) intra-annual anatomical information in the density profiles of *T. superba*, and (ii) the ability to delineate tree-ring boundaries (Chapter 3), a method was tested to exploit intra-annual wood traits assisting the cross-dating process. An important endeavour was to develop a method that runs with density-based objective criteria to achieve a formalisation of cross-dating, avoiding a high degree of “expert knowledge”, therefore going beyond the manual comparison of cores. To provide a benchmark, two temperate species with a known dendrochronology potential, but a differing wood anatomy (*Fagus sylvatica*, diffuse porous vs. *Quercus petraea,* ring porous) were added to the dataset.

In-house developed software routines treat all data from input (tree cores) to output (dated density profiles). Several batch processes are implemented in the toolchain, minimizing manual effort. Moreover, wood density is determined simultaneously by calibration using a reference material. Since many tropical tree species remain poorly studied for their dendrochronological potential, the toolchain allows to screen large sets of cores for such species.

The toolchain enables cross-dating entirely based on batch generated X-ray CT profiles, which is a new approach in dendrochronology. The obtained similarity, i.e. a correlation based similarity criterion of density information, is a robust and objective method in the decision-making process of cross-dating, which can improve tree-ring analysis for species that have a tree-ring boundary characterized by a change in density, but are more difficult to match.

This chapter aims at filling a methodological gap concerning the integrative approach of tree-ring analysis and more continuous proxies such as density profiles and illustrates how an X-ray CT-based toolchain with minimal labour-input results in a digital archive and allows data matching of intra-annual wood traits via density-based pattern matching.

Ring width and density information can be complementary in climate assessment, yet this will depend on the species studied. This also will determine the success of DBPM: if DBPM appears to be better than traditional cross-dating, this implies that the synchronous density signal in this species is higher than the ring width signal (Allen *et al.,* 2012).

Recommendations

High-throughput profiling in combination with exploratory tools such as density-based pattern matching are necessary to establish high-resolution chronologies, complementary to other techniques that allow to incorporate detailed anatomical, chemical and isotope information (Fonti et al., 2010; Schollaen et al., 2014; Hietz et al., 2015) within tree-ring boundaries.

The dated density profiles can be further synchronized at the intra-annual level by means of microstructure alignment of intra-annual profiles once all cores are cross-dated (Bender *et al.,* 2012). However, to achieve this, precise information on cambial phenology is necessary as well (Stangler *et al.,* 2016). Given the high variability in phenology and relative growth as shown in Chapter 2, future work should focus on how cambial phenology and densitometry can be related.

The development of a database of density profiles is an important milestone. In the framework of the wood economics spectrum as discussed by Chave *et al.* (2009), it is

important to extend the existing wood density databases with more detailed information on the radial variation, and especially to add a temporal component to it. Not only does this create the opportunity to build large datasets of digital wood samples, but also to formalize certain concepts of tree-ring research, especially for the vast amount of tropical species for which little expert knowledge is available and a weaker seasonality is present.

On these lesser known species, often occurring in the understory, it is of the essence to start studying phenology:

(iv) Apply the above techniques on multiple species from different functional groups, from the seasonal scale to the multi-decadal scale.

Chapter 5 investigates the intra-annual radial growth of several species of the Mayombe. This is the first study of cambial activity on understory species in tropical Africa, complementing the seasonal diameter measurements from Couralet et al. (2010). Stem disks were digitized to map and track ring phenomena such as wedging. X-ray Computed Tomography imaging proves to visualize the wound tissue with sufficient resolution and image quality for measuring relative growth, and if needed, provides a guided selection for micro sectioning or sanding. We show varying intra-annual growth patterns, which implies species-specific responses on climate. However, these responses can also be influenced by other factors such as site and surrounding trees. As seasonality is weaker in the tropics compared to more temperate regions, the impact of aforementioned factors can be considerable. The striking observation that so many sampled pinnings showed no xylem growth at the stem base, apart from wound-induced local growth, emphasizes the complexity of tropical wood formation and resource availability of these species. There is a need for additional thorough research on phenomena such as patchy growth (Schmitz et al.) and wound-induced wood formation. The aspect of wedging and missing rings is well documented in temperate and arctic regions. However, it is rarely studied in the tropics, though it is being widely acknowledged as a problem in tropical tree-ring analysis. Therefore, pinning and related studies are essential to explain difficulties in tree ring-analyses, especially in the African forests, where many species remain understudied.

Chapter 6 is the first study on tropical trees across several functional groups, which combines repetitive dbh and xylem radial growth measurements with a total time span of 66 years. We used the presence of a tag nail from 1948 as a cambial mark to derive long term radial increment from a historic forest plot. Many of these species are not considered for tree-ring analysis, but do form an important part of the tropical forest ecosystem. As a result, this unique experiment gives us a broader insight in a forest-wide tree response,

rather than the response of trees with “known” dendrochronological potential, often of commercial value. The most striking observation is that the entire dataset shows 32 discernible rings on average, while 66 years have elapsed. In absolute terms there is a difference between growth rates, but the number of missing rings is approximately the same across the different functional groups, with the largest number of missing rings occurring in the understory. The unique availability of nail traces allowed us to assess differing radial xylem growth patterns.

Recommendations

Large and old trees play an important role in the carbon balance of tropical forests (Groenendijk, 2015). Indirect measurements, by means of radiocarbon dating, have estimated tree ages up to 1000 years (Chambers *et al.,* 1998), whereas dendrochronological analysis has shown lower tree ages (Worbes & Junk, 1999). Both methods are flawed, given that uncertainties are inherent to radiocarbon dating and dendrochronology appears to be failing for understory species. Although postulated that missing rings are the premise of death in higher latitudes (Wilmking *et al.,* 2012), it is difficult to support this with experimental evidence, and species might just be genetically adapted to this life strategy of survival without frequent ring formation at the sampled height, or only forming non-periodical rings.

In this respect and to my opinion, the degree of “false positives” using classic tree-ring descriptive statistics such as Glk (Buras & Wilmking, 2015), where, given the formula, spurious correlations occur when applied to time series such as in the tropics, could be considerable in these species. Radiocarbon analysis (Chambers *et al.,* 1998, Vieira *et al.,* 2005) is inaccurate after 17th century and before 1960 (Worbes & Junk, 1999). The unique presence of pre bomb-peak cambial markings presented in this work, is rare, but essential to link xylem traits to radial growth. Moreover, based on abovementioned observations, the oldest individuals are probably not only the big trees, but also the smaller understory ones.

Based on all the above, it is clear that in order to assess and reveal the driving forces of the patterns of wood traits on pith-to-bark cross-sections in tropical trees, a multitude of approaches are necessary to encompass the large variation in the xylem of tropical trees. This work has brought methodological improvements to measure traits as continuous variables from pith to bark, but also acknowledged that phenology still remains a key aspect in order to fix traits on a time axis, from the intra-annual to the multi-decadal level.

Concluding remarks and outlook

This dissertation tackles the need for considering and dating xylem traits at the intra-annual timescale, and expanding this by proxy analysis in a fast and effective way to reveal tropical tree response, within species and between species. Dendrochronology is designed for higher latitudes in order to compute ring width or other single-value-per year variables. However, the complexity of tropical forests, and their subtle inter- and intra-annual climatic variations, necessitate assessing more than ring widths in the xylem. Current available computational power and the rapid development of imaging techniques allow analyzing intra-annual xylem traits. The use of X-ray Computed Tomography densitometry shows to be a suitable tool in this ongoing quest.

Varying cambial phenology between and within species is observed in this study for our reference species *T. superba.* Density-Based Pattern Matching of density profiles is a first step in finding similarity between trees based on intra-annual traits. This correlation measure will match trees that have a similar intra-annual profile, and thus, a similar cambial phenology. Further efforts should be made by including data from cambial pinning experiments, and other criteria for assessing similarity.

The multiscale approach of the X-ray CT densitometry tool allows to create broad trends of density to estimate carbon stocks, up to high-resolution density profiles, which reveal information on intra-annual wood traits which in turn, can increase our knowledge on mechanisms of seasonal growth and phenology.

When mulitidecadal xylem traits are assessed on several trees of an old monitoring experiment from the Nkulapark, phenomena such as missing and non-periodical rings are more the rule than the exception. Furthermore, it was noticed that some trees only form little to no increment after a 66 years monitoring experiment, which has major implications for tree-ring analysis, and should be further investigated through xylem traits. This rare study of combining pre-bomb peak cambial marks and DBH measurements is essential, as traditional tree ring analysis cannot overcome this, permanent sample plot data lack the

accuracy, and radiocarbon is not always suitable for trees older than 50 and younger than 400 years. The phenomena of irregularities of ring formation should not be seen as an anomaly, but rather an opportunity for future research on these species, and revealing their age.

Because of fast data acquisition of density profiles presented here, the database of density profiles should be further expanded with cores from all over the world, providing detailed biomass estimations from pith to bark. Density profiles should therefore be considered in ongoing forest monitoring via permanent sample plots, and flux measurements. Carbon accounting in a detailed way is especially relevant for African forests, and given the current pace of deforestation, it is vital that correct estimations of the current biomass stock and tree growth are quantified, while predicting future stocks under climatic changes.

9

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10

Supplementary information

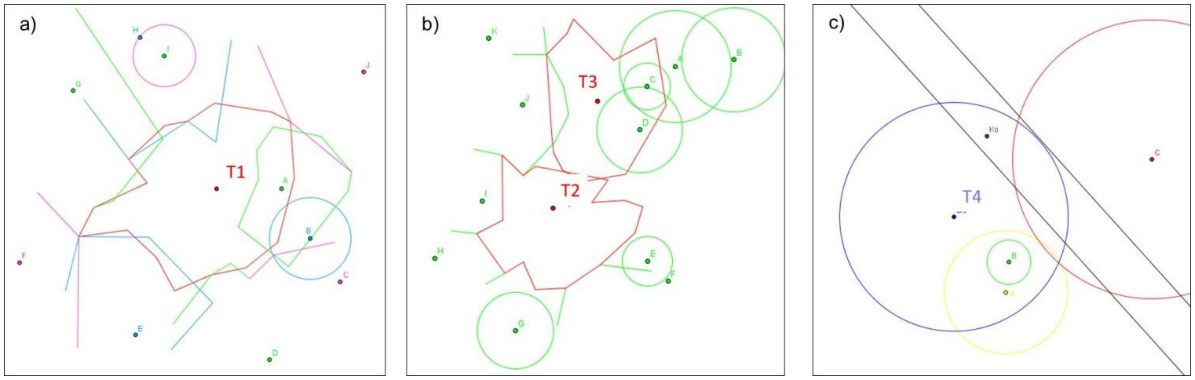


Figure S2.1 Site description of a) tree T1 in a young secondary forest, (b) T2and T3 in an old secondary forest and (c) T4 in an open field. All surrounding trees were measured.

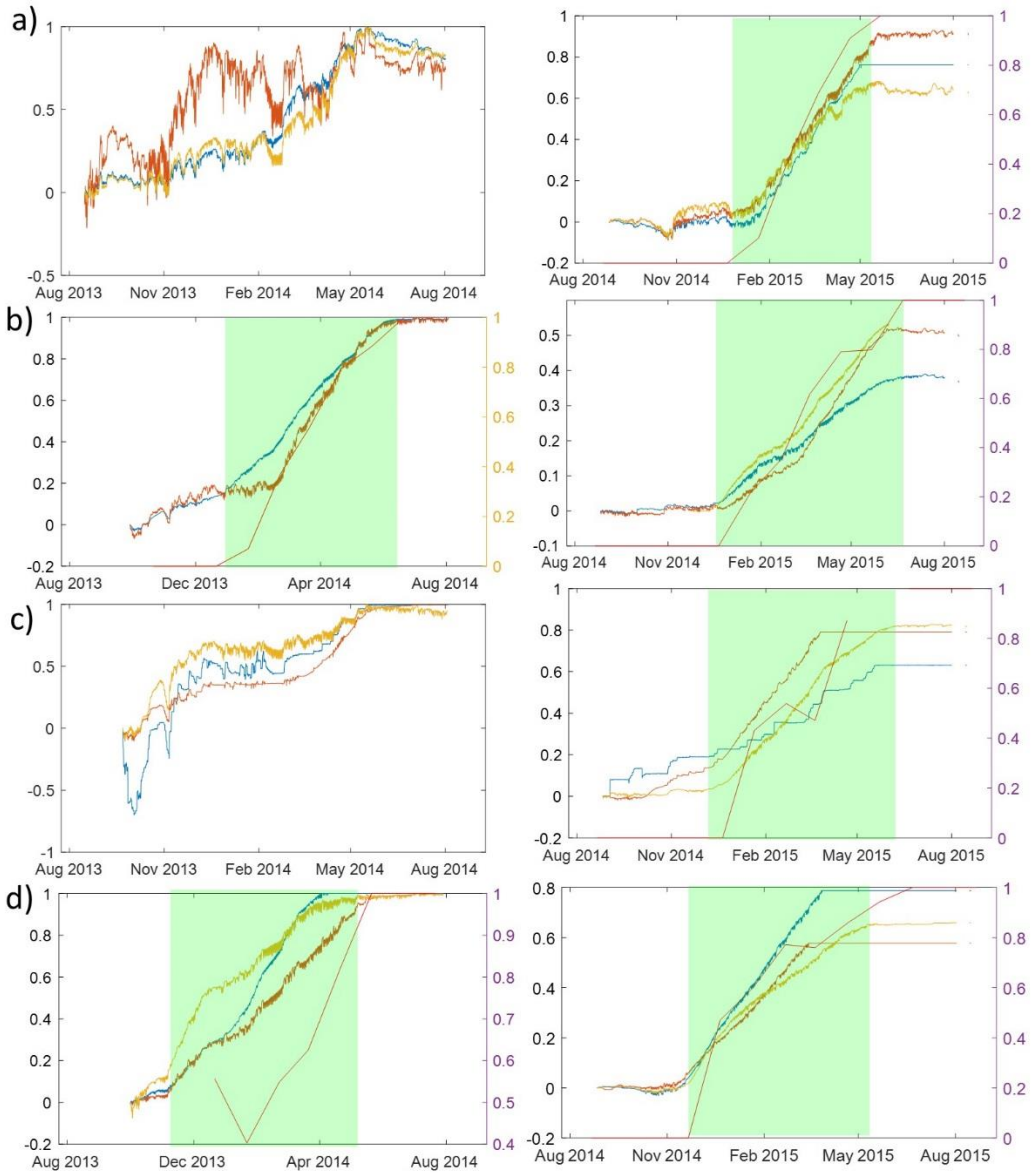


Figure S2.2 SDV series coupled to cambial pinning series. All series were rescaled between 0 and 1 for comparison.

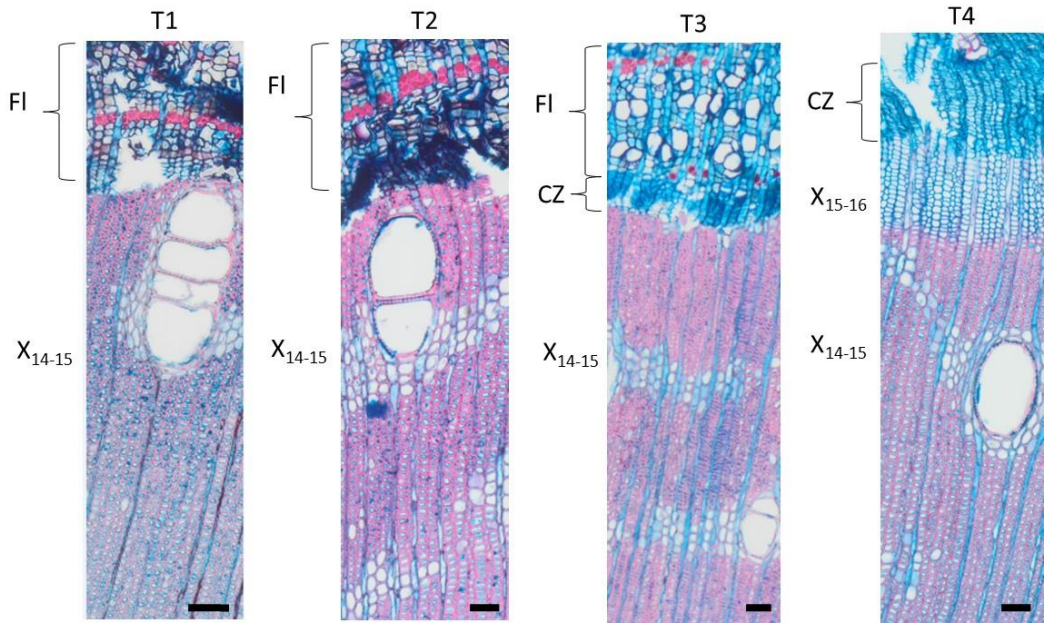


Figure S2.3 Wet sections taken after felling in December 2015, showing only xylem growth in T4. X is xylem, Fl phloem and CZ cambial zone. Scale = 100µm.

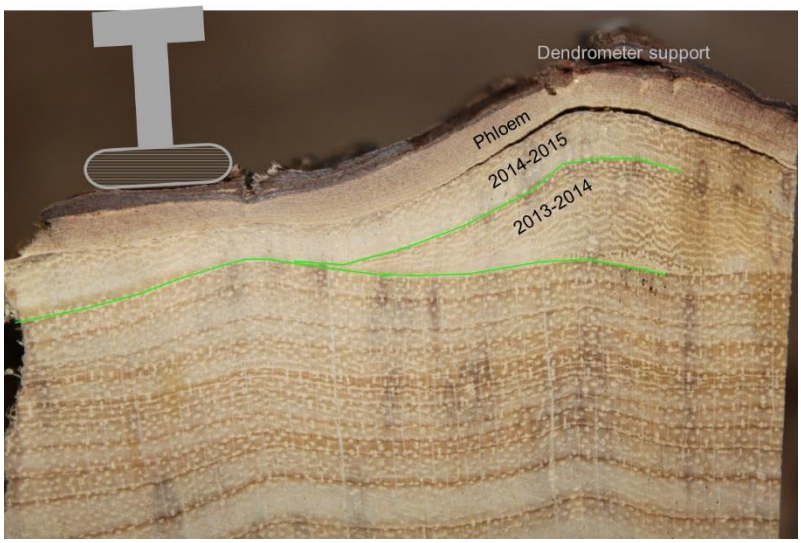


Figure S2.4 Season 2013-2014 showing a missing ring on tree T1 at the dendrometer position. At the dendrometer support a wound-induced ring is formed. In the 2014-2015 a normal tree ring was formed.

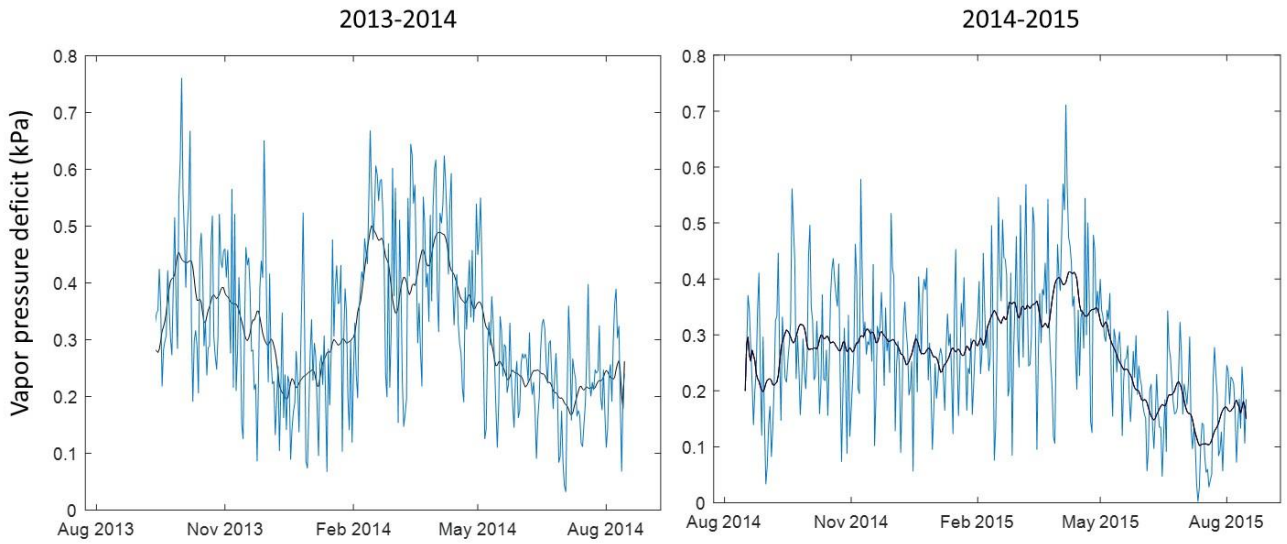


Figure S2.5 Daily (blue) and averaged (black) Vapor Pressure Deficit for the two growing seasons.

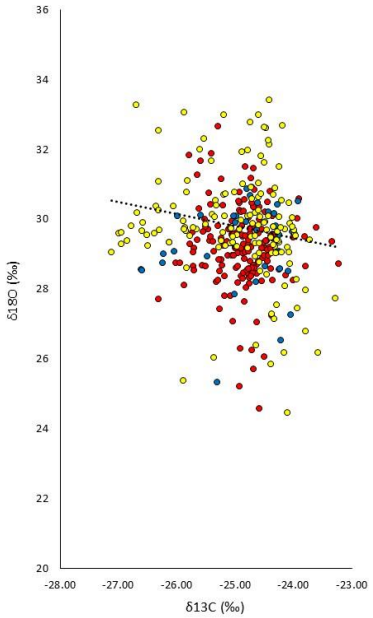
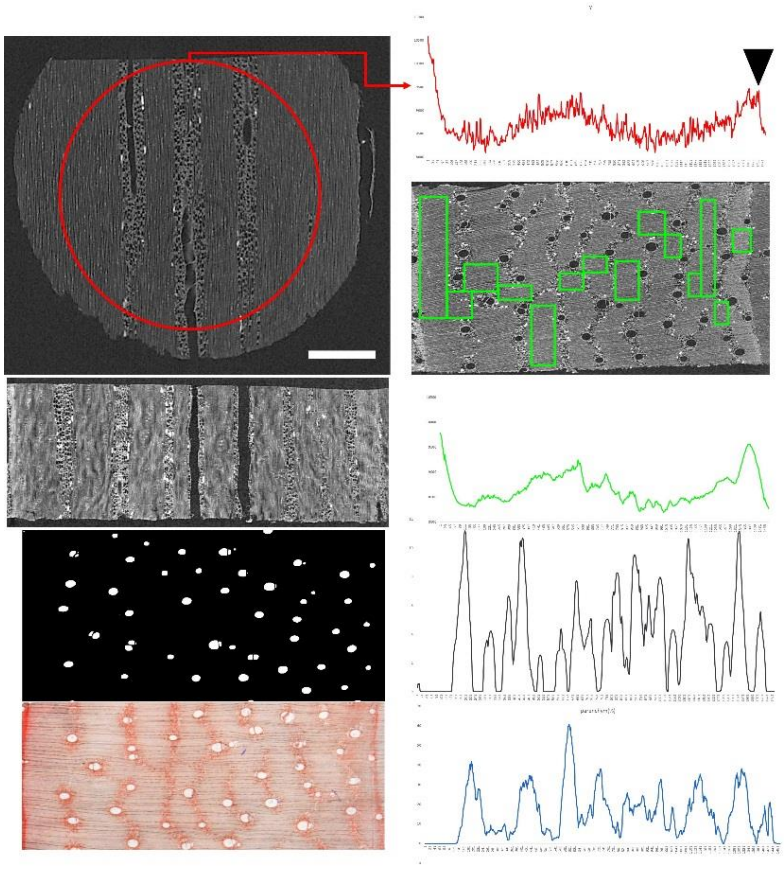


Figure S 2.6 $\delta^{13}C$ and $\delta^{18}O$ are not correlated (R=-0.1337,P=0.0135). Yellow=T2, blue=T3 and Red=T4.



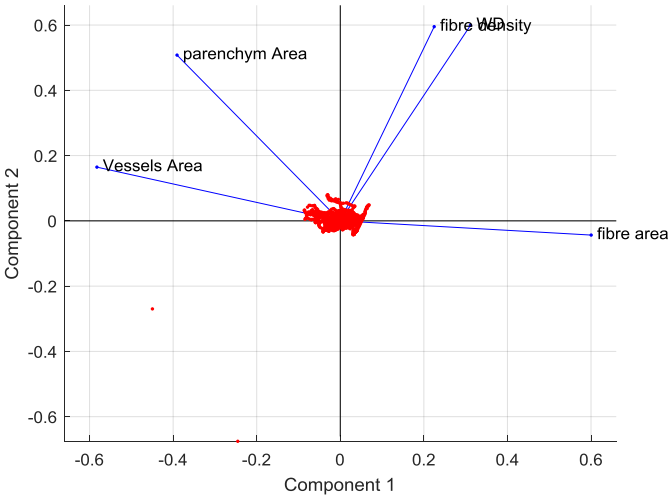


Figure S3.1 Anatomical decomposition of a ring scanned at 4µm, enabling to measure the main anatomical variables. Vessel- and parenchyma fractions can be assessed and separated on high-resolution scans (4µm), which allowed to measure the fibre wall fraction indirectly, through fibre greyscale variation (green rectangles and green curve in Fig 6), of which the pattern concurs with the total intra-annual profile (red curve). Moreover, based on results of Van den Bulcke et al. 2014, this can be performed in an automated way, through eliminating vessels to obtain vessel-free density. PCA analysis shows similar results as obtained above.

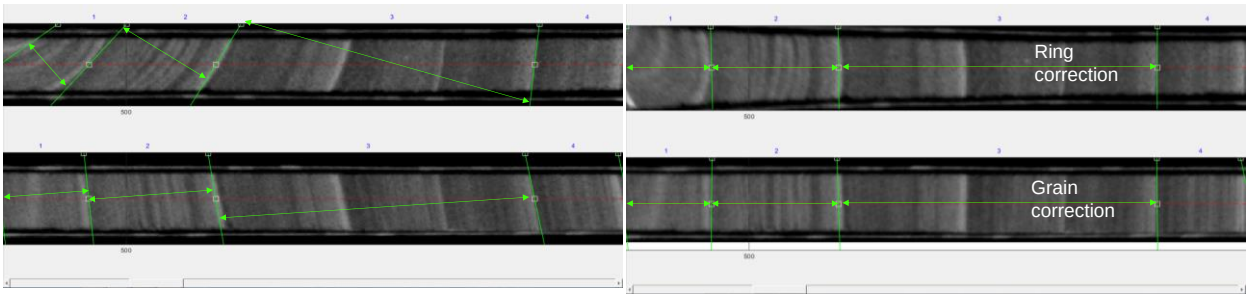


Fig. S4.1 Screenshot of ring and grain angle correction on a Terminalia superba core.

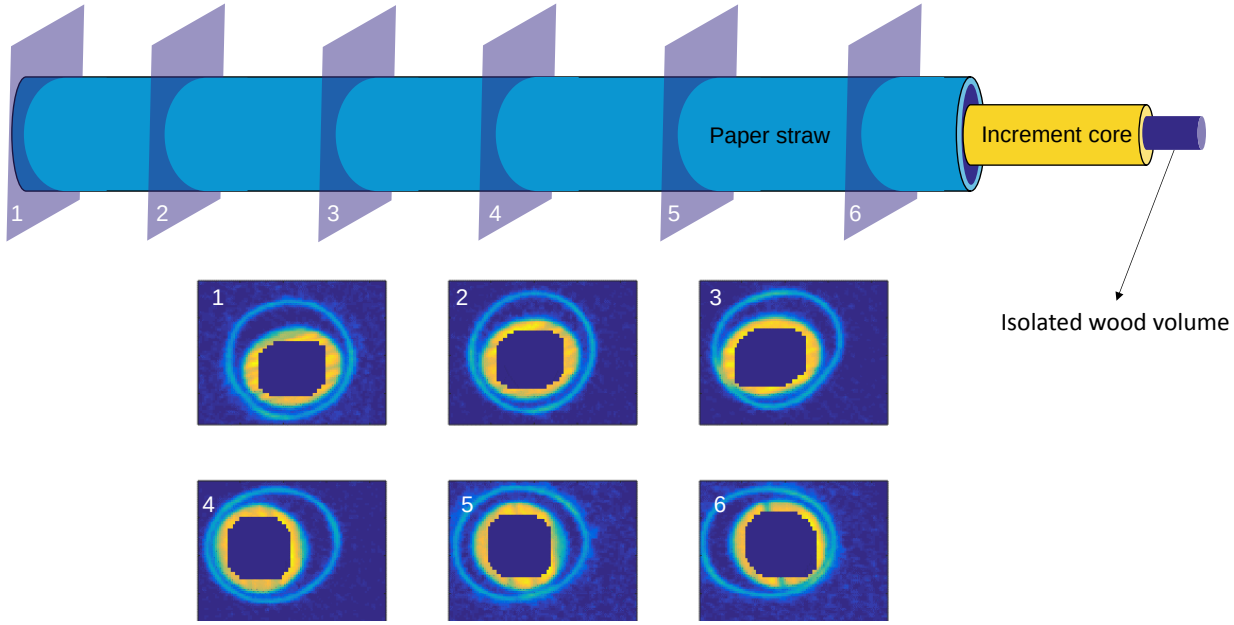


Figure S4.2 Segmenting the wood volume from background for densitometry profiling. Both paper straw and edge effects are automatically excluded during segmentation.

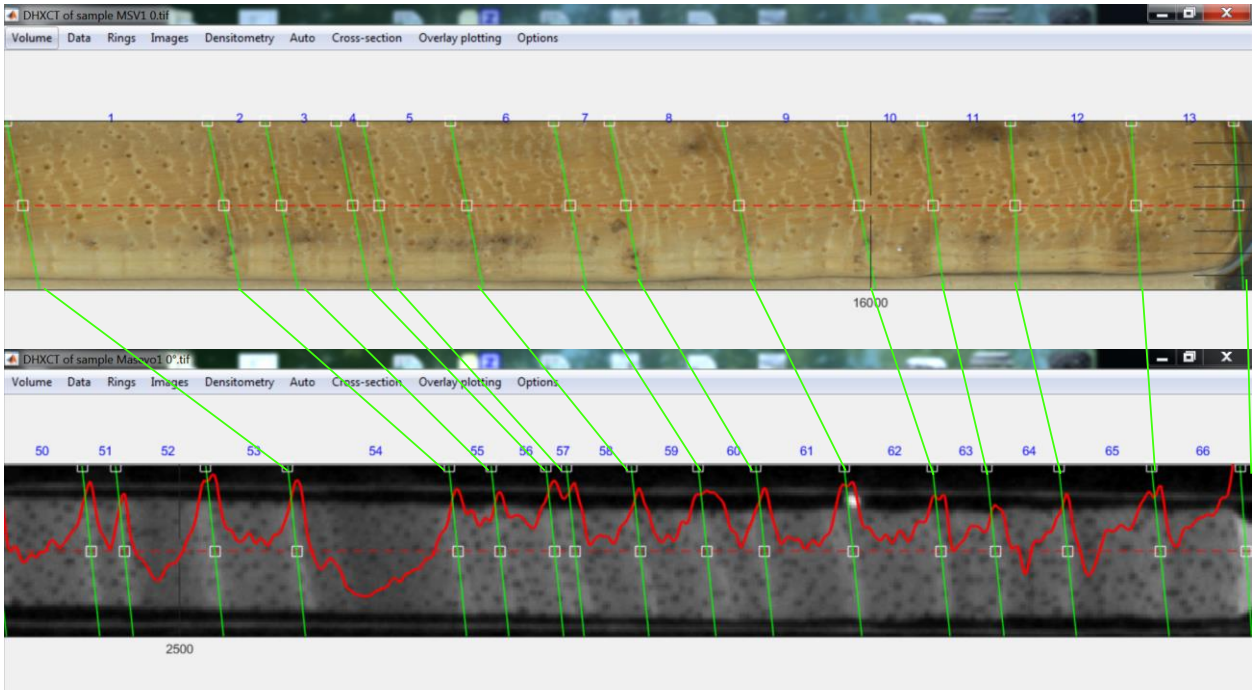


Figure S4.3 The graphical user interface allows to include optical images to complement the X-ray images during tree ring demarcation (Terminalia superba).

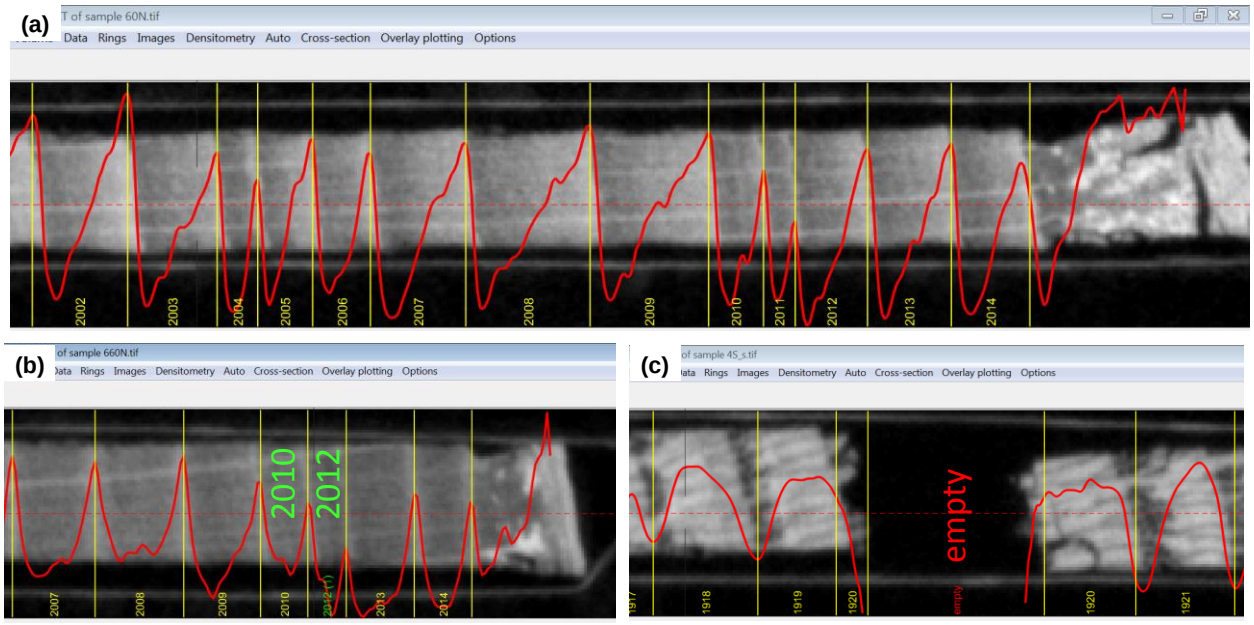


Figure S4.4 (a) Screenshot of the X-ray CT graphical user interface for a Fagus sylvatica core, with density profile, ring boundaries and years in overlay on the X-ray image. Rings can be added or deleted, based on visual inspection or error detection via density-based pattern matching. (b) missing and (c) broken rings can be indicated and are taken into account for further processing.

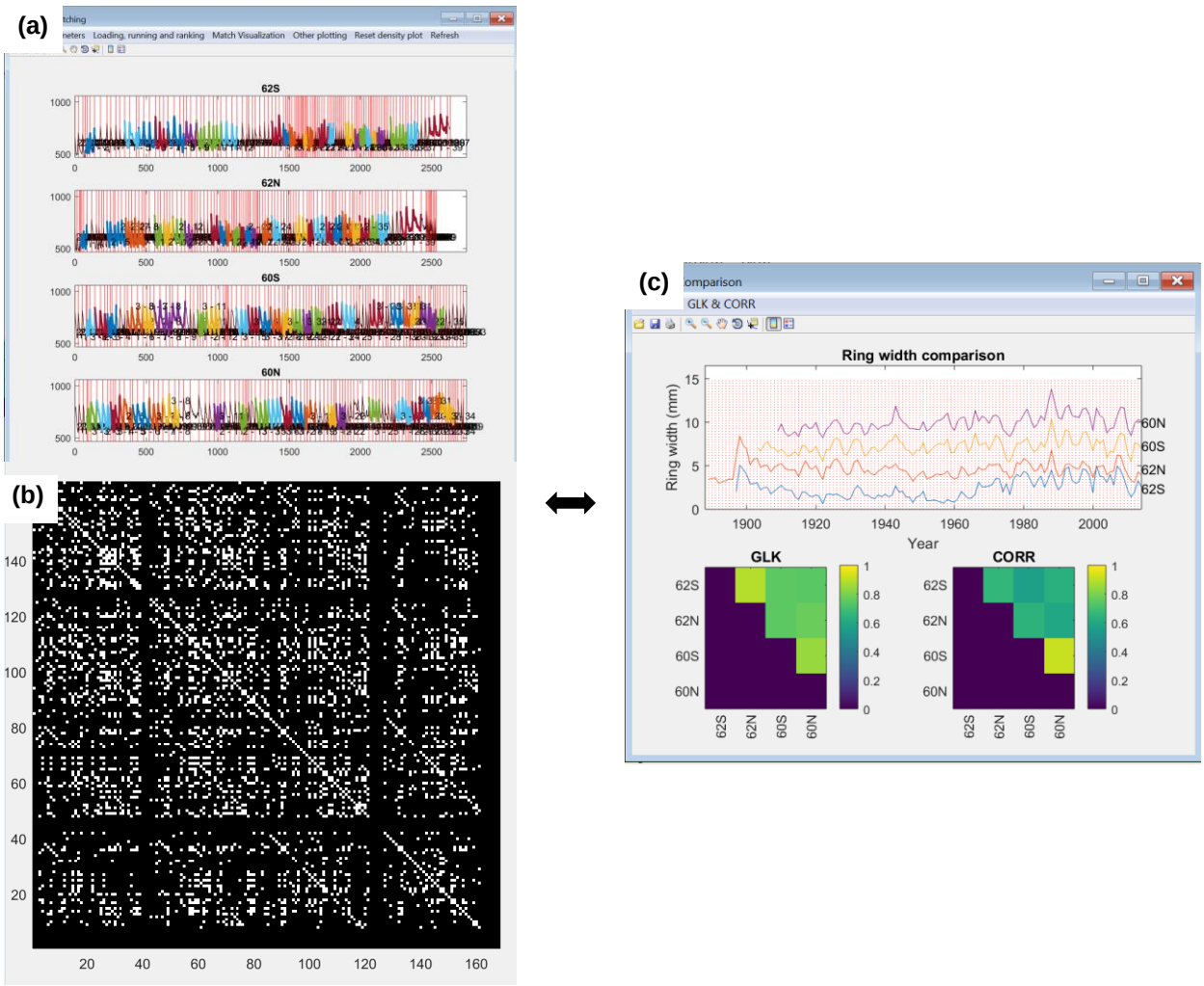


Figure S4.5 Density-based pattern matching can be visualized with (a) plotting of clusters on the profiles and (b) a correlation matrix, and can be verified with (c) classic ring width parameters.

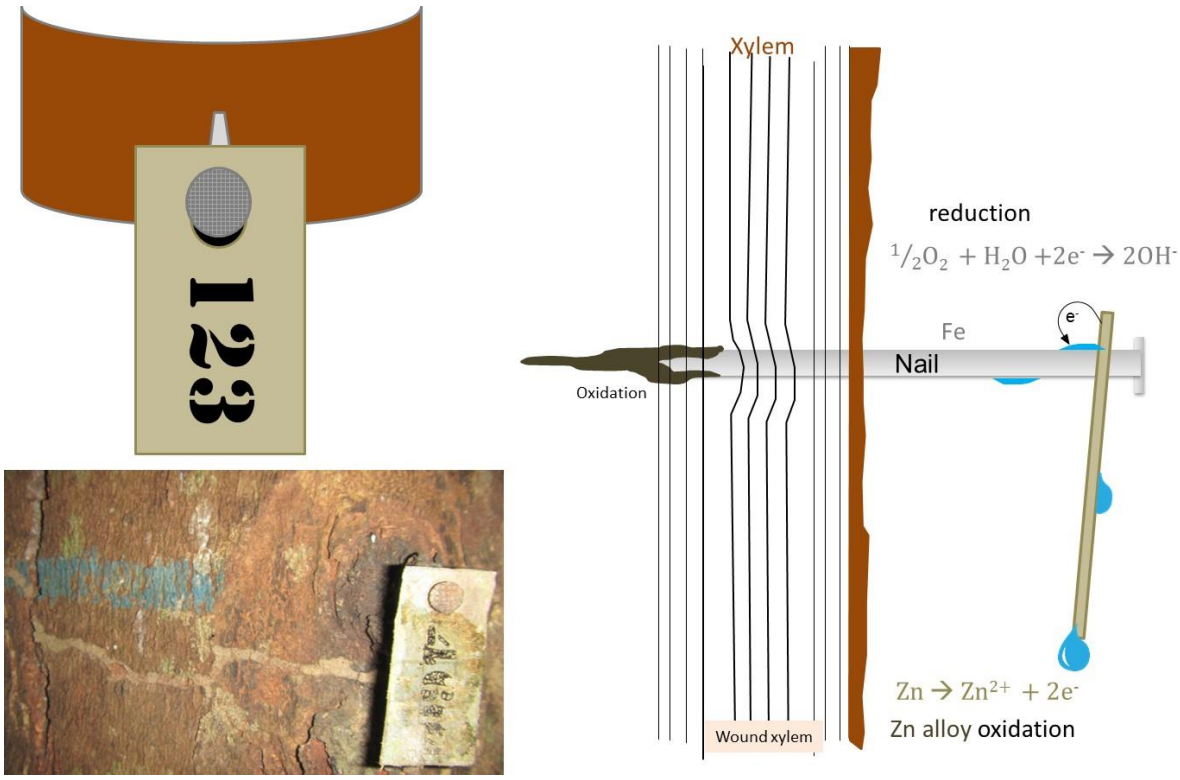


Figure S5.1 The nails remain largely intact after 66 years of exposure. The zinc number tag could have served as a sacrifice anode, which allows cathodic protection of the iron nail when wet.

Table S5.1 Overview of the counted rings per species

Species	N° of trees	Median ring value	Max N° of rings	Mean N° of rings	Standard deviation
Prioria balsamifera	21	63	66	61.06666667	5.470265946
Celtis mildbraedii	5	17	35	20.6	8.142481194
Corynanthe paniculata	7	41	66	44.14285714	14.66774888
Polyalthia suaveolens	3	18	21	18.66666667	2.081665999
Funtumia sp.	5	33	52	34.6	10.87658034
Zanthoxylum gilettii	3	8	15	9.333333333	5.131601439
Isolona dewevrei	1	25	25	-	-
Hexalobus crispiflorus*	1	10	-	-	-
Hylodendron gabunense	2	14	20	14	8.485281374
Allanblackia floribunda	1	25	-	-	-
Microdesmis puberula	1	10	-	-	-
Lannea welwitschii	1	15			
Monodora angolense	1	17			
Terminalia superba*	5	11	17	12.1	3.281259921

* Samples with lobbate growth, or sample between buttresses, see Fig. S. 5.2, are not representative for the radial growth, and were omitted in further analysis.

Figure S5.2 Several aspects of assessing 66 years of tree growth with a) reliable cylindrical stems, b) zero radial increment at the measured height, c) buttresses that ascend the tree trunk and enlarge, causing the normal radial growth from 1948 to be replaced by (b) butresses growth or no growth between the butresses in 2014, which hampers proper measurement of radial increment;d) irregular stems.

11

Curriculum vitae

Personalia

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Driver License: B

Education

- 2012 to 2016: ***PhD student in Applied Biological Sciences: Forest and Nature Management*** at the Laboratory of Wood Technology (Ghent University) and the Royal Museum of Central Africa.
  PhD dissertation: intra-annual to multi-decadal xylem traits in a tropical moist semi-deciduous forest of Central Africa.

- 2010 to 2012: ***MSc Forest and Nature Management*** (faculty of Bioscience Engineering) at Ghent University, Belgium. Degree obtained with greatest distinction.
  MSc dissertation: Structural/chemical and calorific properties of tropical wood species.

- 2007 to 2010: ***BSc Land and Forest Management*** (faculty of Bioscience Engineering) at Ghent University, Belgium. Degree obtained with distinction.

- 2001 to 2007: High school, Koninklijk Lyceum Aalst, Belgium
  Major in Science and Modern Languages

Work experience

- **4 years** as a PhD student at the Laboratory of Wood Technology (Ghent University) and the Royal Museum of Central Africa, conducting experiments, wood microscopic analysis and data analysis

Experience abroad

- A total of **5 months** in the Luki Biosphere forest reserve of the **Democratic Republic of the Congo** (2013, 2014 and 2015), which included leading a team for installing measuring equipment and collecting tree cores for analysis.
- **5 weeks** in **Surinam** for master thesis where sampling was performed and local sawmills were visited to study calorific valorisation of wood residues of various timber species.

Language

	Reading	Speaking	Writing
Dutch (first language)	5	5	5
English	5	5	4
French	5	5	4
German	4	4	3
Spanish	Notions		
Lingala	Notions		

Software

All Microsoft Office related programs (MS Project included), Remote sensing and GIS (IDRISI, Quantum GIS, ArcGis), Math programs and modelling (Matlab), tree ring analysis (TSAPWin), Statistics (S-Plus, SPSS, R), Image processing software (ImageJ, Photoshop, Google Sketchup), Basic knowledge of SAP

Network

Ghent University (Ghent, Belgium), Royal Museum of Central Africa (Tervuren, Belgium), Institut de l'étude et recherche agronomiques (Kinshasa, DR Congo), WWF (Belgium/ DR Congo)

Grants

- Special Research Fund Ghent University (PhD research)
- Leopold III grant (field missions)

A1 publications

- Maes SL, Vannoppen A, Altman J, Van den Bulcke J, Decocq G, **De Mil T**, Depauw L, Landuyt D, Perring MP, Van Acker J, Vanhellemont M, Verheyen K. Comparing three ring-width measurement methods for growth release reconstruction. *Tree-ring Research* (under revision)
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- Defoirdt N, Sen A, Dhaene J, **De Mil T**, Pereira H, Van Acker J, Van den Bulcke J. (
- 2017. A generic platform for hyperspectral mapping of wood. Wood Science and Technology doi:10.1007/s00226-017-0903-z

- Vannoppen A, Maes S, Kint V, **De Mil T**, Ponette Q, Van Acker J, Van den Bulcke J, Verheyen K, Muys B. 2017. Using X-ray CT based tree-ring width data for tree growth trend analysis. *Dendrochronologia* **44**: 66-75.

- **De Mil T**, Angoboy Ilondea B, Maginet S, Duvillier J, van Acker J, Beeckman H, Van den Bulcke J. 2017. Cambial activity in the understory of the Mayombe forest, DR Congo. *Trees - Structure and Function* **31**:49–61.

- **De Mil T,** Vannoppen A, Beeckman H, Van Acker J, & Van den Bulcke J. 2016. A field-to-desktop toolchain for X-ray CT densitometry enables tree ring analysis. *Annals of Botany* **117**: 1187–1196.

Conference presentations

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- Selwin Maginet (2014 - 2015). Intra-annual wood formation in tropical forests: case study on the Mayombe forest, west of the Democratic Republic of the Congo

- Mirvia Angela Rocha Vargas (2015 - 2016). Climate response of *Terminalia superba* from the Mayombe forest (Democratic Republic of the Congo): intra-annual stable isotope analysis in tree rings